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A thesis for the Degree of Doctor of Philosophy

**Antioxidant, antimicrobial, and anticancer
effects of Korean propolis**

**한국산 프로폴리스의 항산화, 항균, 항암
효과에 관한 연구**

February 2015

**Department of Agricultural Biotechnology
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**UNDER THE DIRECTION OF ADVISER YOUNG-JOON AHN
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF**

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ABSTRACT

Propolis is a sticky material made from plant growth point protection secretion and resin which are collected by bees, then mixed with bee saliva enzyme, it is used to keep bee colony safe by applying inside of bee hive, and it is consisted of about 50% of resin and aromatic, 25% of bee wax, 10% of essential oil, pollen and mineral. In this study, extraction characteristics of propolis by ethanol were confirmed because of its major use as a food, propolis yield and its active ingredient total flavonoid content were better with more than 70% ethanol concentration. Propolis can be extracted with more than a day extraction time and above 4°C temperature condition.

Propolis samples were collected every two weeks, their amount and ingredient composition changes over time were examined, summer (early June-late August) time collected propolis sample occupied 87% of total 100g collection. Total flavonoids content (7~8%) and total phenolic content (16~20%) of collected samples were maintained without significant differences.

These results confirm the total flavonoid content in propolis samples collected from different regions in Korea, most samples showed 5% or more except Jeju sample (less than 1%). Total phenolic content in propolis was more than 20% with samples from central and southern regions, Jeju samples showed about 15%.

The content of the amino acids in propolis was examined and various amino acids were observed. Heavy metal content of propolis showed that lead content was below the baseline (5ppm), Arsenic and mercury were not detected at all. Minerals (Zn, Cu, Ni) required for the metabolism were contained by a very small amount. Crude lipid contents in propolis of central

and southern regions samples showed average 38%, while Jeju sample exhibited 24%. The main substances (Gallic acid, naringenin, quercetin, apigenin, chrysin, galangin) in propolis were confirmed through HPLC analysis of collected propolis samples by comparing with standard product.

All Korean propolis samples showed excellent antimicrobial effect against bacteria causing stomach ulcers (*Helicobacter pylori*), also exhibited excellent antibacterial activities against caries causing bacteria (*Streptococcus mutans*). The results confirm the proliferative effect on intestinal bacteria (lactic acid) exhibited a high effect when the concentration is low.

The antioxidant effect of propolis was confirmed in a number of ways showing the higher the antioxidant effect by rise of propolis concentration, but concentration of 1000 μ g showed rather poor result. In vivo test, glutathione content was increased, lipid peroxidation was decreased, and the increase of propolis concentration exhibited higher effect.

The experiment using nude mouse to examine anti-cancer effect of propolis was conducted, propolis ingestion result after tumor induction was determined by monitoring body weight and tumor size change, tumor size showed a tendency to decrease with propolis concentration rise.

Antioxidant effect of propolis is common, but propolis has excellent antimicrobial activity against bacteria causing stomach ulcers (*Helicobacter pylori*). With gastric cancer cell lines, propolis lowered the cell proliferation ratio, also reduced tumor size. Propolis can be developed to health functional food which has stomach protection effect as well as antioxidant effect.

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**Keywords : Propolis, antioxidant effect, antimicrobial effect,
anti stomach cancer effect.**

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I . Introduction

The propolis is a Greek word meaning the material which protects the honey bee colony safe, "pro' means protection and "polis" means city. Propolis is a sticky material made from bee collected plant growth point protection secretion and resin mixed with bee saliva enzyme.

It is used for keep safty of bee colony by applying inside of bee hive and has various color including dark brown and yellowish brown. Propolis is sticky at warm condition but becomes hard at cool condition, so it is also called "bee glue" (James Fearnley, 2011). Propolis is an oily material collected by worker bees from various plants which secrete substances for protecting growing point and preventing microbial infection of damaged bark. The collected resin is mixed with nursing bee secreted enzyme which works as general antibiotics to bacteria and fungi, then this mixture becomes an effective gluey material named propolis which is a misterious natural antibiotics given by nature (Ghisalberti, 1979).

Bees use propolis for colony protection to prevent fungal and viral infection by applying it on the contamination susceptible surface, to make hive water proof by filling cracks with propolis and to block hive from the

outside. Also propolis is used for hive repair, entrance size adjust, antiseptic for sealing large sized insect intruder which is hard to move outside and preventing decay of carcass, and controlling disease and microorganism growth in the hive. But the most important use of propolis is larvae protection by coating thin layer of this on brood cell for keeping eggs and larvae from microbe before oviposition of queen bee, and a little amount of propolis is mixed with bee wax for brood cell sealing. The plant resin and bee salivation both contain antibiotics, so use of propolis can reduce infection of growing bee larvae and microbe growth in dead animal tissue.

Propolis is a composite material consists of various ingredients such as resin and aromatic (45~55%), bee wax (25~35%), volatile essential oil (10%), pollens, mineral (5%), tannins, and bee secretion & enzyme (Moreno *et al.* 2000).

Propolis is an oily material which is more dissoluble to other solvents than water. In this paper, extraction characteristic of propolis by ethanol because of the major use of propolis as an edible material, its ingredient difference by collection time, peculiar characteristics by analyzing total flavonoid content and other ingredients of samples from various region using HPLC and LC/MS were examined. Also antibiotic and anti-oxidation effects of propolis were identified, antibiotic effect to *Helicobacter pylori*, suppression effect to stomach cancer cell line and stomach cancer cell formation were analyzed.

II. Literature Review

The propolis is generally a sticky natural substance collected and transformed by bees for fill the holes or cracks in hive, it softens inner wall of hive, protects foreign enemy intruding, prevent inside decay of carcass from the outside (Brumfitt *et al.*, 1990; Burdock, 1998). Propolis had been used from ancient because of its beneficial characteristics; it was used in mummification in ancient Egypt (Grange and Davey, 1990). It also was utilized as popular medicine for curing various diseases in 20th century (Castaldo and Capasso, 2002). The origin of propolis is depend on the plants which bee collect resin, Burdock(1998) referred the general plant species, poplar in temperate region, betula in north region, delchampia in equatorial region, clusia in Venezuela, xanthorrhoea in Australia. In temperate region, the origin of propolis in poplar species, identified major ingredients are pinocembrin, pinobanksin and 3-O-acetate, chrysin, galangin and caffeates (benzyl, phenylethyl, prenyl) etc (Bankova *et al.*, 2000).

The ingredients of propolis are more than 200 because of the diversity of plant species and collection season (Ghisalberti, 1979; Greenaway *et al.*, 1991; Marcucci, 1995; Bankova *et al.* 2000; Pietta *et al.*, 2002; Kumazawa *et*

al., 2004; Isla *et al.*, 2005; Silici and Kutluca, 2005; Gomez-Caravaca *et al.*, 2006; Mohammadzadeh *et al.*, 2007; Jasprica *et al.*, 2007; Alencar *et al.* 2007).

The polyphenolic component and flavonoids in propolis show significant anti-oxidation effect, there are several studies referring correlation between anti-oxidation effect and polyphenolic composition in propolis (Bors *et al.*, 1990; Heim *et al.*, 2002; Russo *et al.*, 2002; Kumazawa *et al.*, 2004), and these activity get synergy effect by complex reaction between phenol compound and resin type materials (Burdock, 1998; Markham *et al.*, 1996).

Several studies are being conducted on caffeic acid phenethyl ester (CAPE) contained in propolis and its strong anti-oxidation effect is known. Those studies are focusing anti-oxidation effect (Russo *et al.*, 2002), possibility of neurotransmitter (Lee *et al.*, 2007), NF-kB suppression effect (Choi & Choi, 2008; Lee *et al.*, 2008; Ha *et al.*, 2009; Lee *et al.*, 2010), cancer cell formation obstruction effect (Jin *et al.*, 2005; Parlakpınar *et al.*, 2005; Hwang *et al.*, 2006), anti-cancer effect of and Artepillin C separated from Brazilian propolis (Jin *et al.*, 2005; Parlakpınar *et al.*, 2005; Hwang *et al.*, 2006). And recovery effect on UVA, UVB damaged skin cell (Wu *et al.*, 2011) and cancer cell formation control effect (Ha *et al.*, 2010; Bae *et al.*, 2011) of chrysin are also being studied.

Antibiotic effect of galangin is mainly studied (Pepeljnjak & Kosalec,

2004; Cushnie *et al.*, 2007) and its function as a anticancer medicine candidate is also under study (Heo *et al.*, 2001, Shafat *et al.*, 2000, Sohn *et al.*, 1998). Antivirus effect of quercetin was identified by Debiaggi *et al.*(1990).

There are diversified studies under progress to analyze propolis, many analysis techniques are being used for analyzing characteristics of phenolic contained in propolis (Bankova *et al.*, 2002; Popova *et al.*, 2004; Watson *et al.*, 2006), most general methods are HPLC and LC/MS (Volpi and Bergonzini, 2006; Gardana *et al.*, 2007; Falcão *et al.*, 2010; Pellati *et al.*, 2011), and MS/MS is used to analyzed structural characteristics (Cuyckens and Claeys, 2004).

Many researchers studying propolis reported its functional characteristics show significant difference by collected region (Banskota *et al.*. 1998; Burdock, 1998; Hideki *et al.*, 1998; Kujumgiev *et al.*, 1999; Rosalen *et al.*, 1999; Gregoris, *et al.*, 2010; Hegazi *et al.*, 2000; Sforcin, 2000; Velikova *et al.*, 2000; Sorkun *et al.*, 2001; Park *et al.*, 2002; Kumazawa *et al.*, 2004).

Propolis is globally known as a natural antibiotics, so its antibiotic effect to various microorganisms are under study, one of these research focused on antibiotic activity of propolis to microorganisms in oral cavity (Koo *et al.*, 2000; Santos *et al.*, 2002), and the propolis extract showed suppression of microorganism multiplication. When the oral fibroblast from healthy persons

was artificially infected by pathogen, application of propolis suppressed fibroblast and increase of pathogen (Rodriguez *et al.*, 1997), Also another study reported ingestion of propolis alleviated the inflammation of 27 person's oral abrasion (Magro *et al.*, 1994).

Various studies are being conducted for analyzing effects of propolis such as antiviral (Amoros *et al.*, 1992; Serkedjieva *et al.*, 1992; Amoros *et al.*, 1994; Kujumgiev *et al.*, 1999; Vynograd *et al.*, 2000; Ito *et al.*, 2001; Huleihel *et al.*, 2001; Huleihel and Isanu, 2002; Gekker *et al.*, 2005), anti-fungal (Dobrowolski *et al.*, 1991; Dimov *et al.*, 1991; Kujumgiev *et al.*, 1999; Murad *et al.*, 2002; Sforcin *et al.*, 2001), parasitic insect extermination (Higashi and De Castro, 1994; De Castro and Higashi, 1995; Salomão *et al.*, 2004; Freitas *et al.*, 2006), anti-inflammatory (Wang *et al.*, 1993; Strehl *et al.*, 1994), anti-cancer (Ikeno *et al.*, 1991; Matsuno, 1995; Kimoto *et al.*, 2001), liver protection (Gonzales *et al.* 1995, Basnet *et al.*, 1996), immune control (Dimov *et al.*, 1991), anti-cancer effect by suppressing new blood vessel formation in tumor (Ohta *et al.*, 2008), growth control of cancer cell line (El-khawaga, 2003).

The disease suppression effect of propolis is induced from anti-oxidation (Arjun *et al.*, 2000; Fang *et al.*, 2000; Isla *et al.* 2001; Russo *et al.*, 2002; Kolankaya *et al.*, 2002; Hamasaka *et al.*, 2004; Kumazawa *et al.*, 2004; Ozen

et al., 2004; Shimizu *et al.*, 2004). By these effects propolis is extensively used for anti-inflammatory, anti-diabetic, heart disease improvement, anti-cancer and health improving food and beverages (Banskota *et al.*, 2001; Burdock, 1998).

III. Material and Method

1. Extraction characteristics of propolis by ethanol

1.1. Propolis yield by ethanol concentration

Weighed 5 gram of propolis sample from Suwon, Daegu and Jeju respectively, ethanol (HPLC grade, Fisher, USA) dilutions with various concentration (0~100%) were prepared by diluting with triple distilled water, then propolis samples were mixed with ethanol by 1:10 volume, and digested for 48 hour at room temperature. After digestion, samples were filtered (Whatman #2 paper), concentrated and yield was measured.

1.2. Propolis yield by extraction time

Weighed 5 gram of propolis samples from Suwon, Daegu and Jeju respectively, diluted with ethanol 1:10 volume, digested with different time. After digestion, samples were filtered (Whatman #2 paper), concentrated and yield was measured.

1.3. Propolis yield by extraction temperature

Weighed 5 gram of propolis samples from Suwon, Daegu and Jeju respectively, diluted with ethanol 1:10 volume, digested for 48hour at freezing (-20°C), cold (4°C) and room temperature (25°C) condition. After digestion, samples were filtered (Whatman #2 paper), concentrated and yield was measured.

1.4. Propolis extraction by sonication

Weighed 5 gram of propolis samples from Suwon, Daegu and Jeju respectively, diluted with ethanol 1:10 volume, treated with ultrasonic cleaner (Branson 8510, USA) for 100 min at room temperature. After treatment, samples were filtered (Whatman #2 paper), concentrated and yield was measured.

1.5. Total flavonoid content analysis in propolis

Weighed 0.1g of extracted and concentrated propolis, dissolved with 80% ethanol 20 ml, then centrifuged (3,000 rpm, 10 min). Then supernatant was collected, residue was extracted 3 times with 80% ethanol, then all extracts were united to one sample and 80% ethanol was added to make 50 ml sample.

Put 0.5 ml of sample into test tube, added ethanol 1.5 ml, 10% aluminum nitrate (Sigma, USA) solution 0.1ml, water 2.8 ml, stirred sufficiently and

stationed for 40 min. Another process which aluminum nitrate solution was substituted with 0.1 ml water was finished. Absorbance of both sample fluid bed was measured using 10mm cell with 415 nm wave length using water as control. Using the value by subtracting latter process absorbance value from former one, then total flavonoid content (mg/ml) was calculated using calibration curve acquired by quercetin (Sigma, USA).

$$\text{Total flavonoid contents(\%)} = \left(\frac{\text{mg}}{\text{ml}} \right) \times \frac{50\text{ml}}{\text{weight of sample(g)} \times 1,000 \text{ (mg)}} \times 100$$

1.6. Total phenolic content analysis

Total phenolic content was measured using modified Folin-Ciocalteu method (Kuyala *et al.*, 2000). Sample solution 0.5ml (three times extracted with 80% ethanol) was mixed with 0.5ml of 1N Folin-Ciocalteu (Sigma, USA) solution 0.5 ml and 0.5ml of 10% Na₂CO₃ solution, then stationed for 50 min. Sample was centrifuged at 150G for 10 min, absorbance of supernatant was measured by UV/VIS Spectrophotometer (Perkin-Elmer Lambda 10, USA) at 760nm wave length. Total phenolic content (mg/ml) was calculated using calibration curve acquired by gallic acid (Sigma, USA).

2. General characteristics of propolis by collection time and origin (Korean & foreign)

2.1. Total flavonoid and phenolic content analysis of propolis collected by time

From the middle of May, propolis samples were collected by 2 week interval by installing collection net at the hives of experimental apiary located NAAS, seodun-dong, Suwon, Korea.

Propolis samples from each collection were extracted using 80% ethanol at 1:10 volume ratio, and total flavonoid & phenol contents were measured using HPLC. For HPLC (AKTA explorer 10, Amersham Pharmacia Biotech, Sweden), ODS C18 column (Merck, Germany) was used, dispersive solvents were 5% formic acid and methanol, the process initiated with 30% methanol and sustained 30 min, then increased with 80% methanol, and sustained 40 min.

2.2 Propolis collection

For the analysis of propolis, samples were collected from domestic region, and foreign propolis samples were collected for comparison.

2.3. Ingredient analysis

2.3.1. Total flavonoid content (Health Functional Food Act, MFDS Korea)

2.3.2. Total phenolic content (Folin-Ciocalteu method)

2.3.3. Amino acid content analysis in propolis

Each 5mg of extracted propolis samples were dissolved with 0.5ml 80% ethanol, then 0.5ml of 12N HCl was added, and dissolved by putting samples into vacuum hydrolysis tube (Kontes, Vineland, NJ). Residual oxygen was exchanged by injecting nitrogen gas. After closing tube lid hydrolysis was conducted at 110°C for 24 hours. Solvent was removed under vacuum, filtered sample with 0.45um filter, and amino acid content was measured with amino acid analyzer (Amersham Pharmacia Biotech, Biochrom 20 Plus type amino acid analyzer, Sweden).

2.3.4. Color of propolis collected from different regions

Hue, value and chroma of each extracted propolis sample were measured using color-difference meter (Nippon Denshoku, Japan).

2.3.5. Heavy metal content of propolis

Heavy metal and mineral content of extracted propolis samples was measured three times by ICP-MS (Agilent 7500a, USA).

2.3.6. Crude lipid content of propolis

Extracted propolis sample 3g was weighed, put into Soxhlet extraction apparatus, extracted fat with ether, crude fat content was measured by collecting and weighing ether (Poon *et al.*, 1956).

2.4. HPLC analysis of flavonoids in Korean propolis

For identification of flavonoids, HPLC-ODS C18 column was used with 5% formic acid and methanol as deploy solvent. For content analysis, AKTA explorer 10 (Amersham Pharmacia Biotech, Sweden) was used.

2.5. Investigation and identification of propolis index material

To investigate and separate propolis index material, sample was deployed using JAIGEL-GS310 column (ZAI instruments, Japan), graduated by prep-HPLC (Z 9150, ZAI instruments, Japan). Flavonoids (Quercetin, Galangin, Chrycin, Rutin) and phenolics (Gallic acid, Caffeic acid, Cinnamic acid, CAPE) were identified using HPLC and LC/MS (MSQ, thermo instrument, USA).

3. Functional effects of Korean propolis

3.1. Investigation of functional property range

To determine the functional property range of the extracted propolis, it was measured spectrum of propolis with 200~500 nm range using UV-VIS spectrophotometer (Lambda 10, Perkin-Elmer, USA).

3.2. Antimicrobial effects

3.2.1. Antimicrobial effects of intestinal microflora

To examine antimicrobial effect of propolis, *Bifidobacterium longum* KCCM 11953, *Bifidobacterium adolescentis* KCCM 11206, *Escherichia coli* KCCM 70089, *Helicobacter pylori* strains were purchased from KCCM. *E. coli* was cultured on LB media under aerobic condition and *Bifidobacterium* species were incubated in an anaerobic condition, the growth inhibitory effect was determined by paper disc method. *Helicobacter pylori* strains were cultured on Brucella Agar containing 5% bovine calf serum and 10ug/ml of vancomycin, 5ug/ml of polymyxin B, 5 ug/ml of trimethoprim, and 2 ug/ml of amphotericin B. The plates were incubated at 37°C for 2 days in anaerobic chamber (Hirayama, Tokyo, Japan), the growth inhibitory effect was determined by paper disc method.

3.2.2. Identification of antimicrobial effect on various harmful microflora

In order to investigate the antimicrobial effects of the various harmful bacteria, strains as oral bacteria (*Streptococcus mutans* 3065, 3289, KCCM 11823), athlete's foot fungi (*Candida albicans*, KCCM 11282, *Trichophyton mentagrophytes*, KCCM 60027, *T. rubrum*, KCCM 60443 *T. ferrugineum*, *Epidermophyton floccosum*, KCCM 11667), methicillin resistant *Staphylococcus aureus* (MRSA) and *Bacillus subtilis* (KCCM 11316), it was confirmed by the disc diffusion method. *Streptococcus mutans* was cultured on brain heart infusion agar media under anaerobic condition *Candida albicans*. *Trichophyton mentagrophytes* was cultured on Emmons modification of Sabourauds agar media under anaerobic condition. *T. rubrum*, *T. ferrugineum*, *Epidermophyton floccosum* was cultured on Sabourauds agar media under anaerobic condition.

3.2.3. Examination of propagation effect on beneficial intestinal microbe

For the examination of propagation effect on beneficial intestinal microbe the number of cultivated *Bifidobacterium longum* was counted using pour dilution plate technique.

3.3. Anti-oxidation effect of propolis

3.3.1. Anti-oxidation effect of propolis in vitro

3.3.1.1. Free radical scavenging effect by DPPH method

Using extracted propolis, samples with 10, 50, 100, 500, 1000ug concentration were prepared and that of 2,2-Diphenyl-1-picrylhydrazyl (DPPH, sigma, USA) was 0.2 mM. DPPH solution 1 ml was mixed with 2ml sample, stationed 10 min, and absorbance was measured at 517 nm wavelength using UV-VIS spectrophotometer (Lambda 10, Perkin-Elmer, USA). Free radical scavenging effect was calculated using following formula.

$$\text{Radical scavenging effects (\%)} = 100 - (A/B \times 100)$$

A: sample absorbance (517 nm)

B: blank absorbance (517 nm)

3.3.1.2. OH radical scavenging activity of 2-Deoxy-D-ribose

Hydroxyl radical scavenging effect of extracted propolis was measured using 2-deoxyribose oxidation method. Phosphate buffer (pH 7.4) 1.2 ml with 100 mM concentration, 0.2 ml of 10 mM 2-deoxy-D-ribose (1.3413g/1L), 0.2 ml of dissolved 0.01 mM FeSO₄ in 3 mM EDTA, 0.2 ml of 10 mM H₂O₂ (0.34g H₂O₂/1L), and 0.2 ml of sample, total 2 ml was mixed and reacted for 4 hour at 37°C. TCA (trichloroacetic acid) 2.8% solution 1 ml was added to this solution for stopping reaction, 1% TBA (thiobarbituric acid) 1 ml was added, heated for 10

min at 100°C and quenched with ice. Absorbance of this solution was measured using UV-VIS spectrophotometer (Lambda 10, Perkin-Elmer, USA) at 532 nm.

$$\text{OH radical scavenging activity} = (1 - (Abs - Abo) / (Abc - Abo)) \times 100$$

Abo: absorbance of no treatment at 532 nm

Abc: absorbance of treated control at 532 nm

Abs: absorbance of sample at 532 nm

3.3.1.3. Radical scavenging effect of Ferric thiocyanate

Extracted propolis sample was dissolved in 80% ethanol, 120 ul of this solution was mixed with 2.51% linoleic acid 2.88 ml and 40 mM phosphate buffer (pH 7.0) 9 ml for reaction. Ethanol 97 ml (75%), 30% ammonium thiocyanate 100 ul, 100 ul of 20 mM FeCl₂ dissolved in 3% HCl were added to 120 ul of mixed solution, stationed 3 min at room temperature, then absorbance was measured using UV-VIS spectrophotometer (Lambda 10, Perkin-Elmer, USA) at 500 nm.

$$\text{Inhibition (\%)} = (A_0 - A_1 / A_0) \times 100$$

3.3.1.4. Anti-oxidation effects using soybean lipoxygenase method

Propolis extract 20 ul, soybean lipoxidase type V 30 ul and 2 ul of 100 mM tris buffer (pH 8.5) were mixed, stationed 2 min at room temperature, and absorbance was measured using UV-VIS spectrophotometer (Lambda 10, Perkin-Elmer, USA) at 234 nm.

$$\% \text{ inhibition} = (A-B)/A \times 100$$

A: control

B: sample

3.3.2 Anti-oxidation effect of propolis in vivo

3.3.2.1. Total glutathione content

Livers of propolis treated and non-treated animal (rat) were extracted, samples (each 0.5g) were homogenized with 9 times weight of 10 mM sodium phosphate buffer (pH 7.0). From this homogenized solution, 0.5 ml was taken, 3 ml mix of 1% phosphoric acid and 0.6% TBA was added to sample, heated for 45 min, n-butanol 4 ml was added and obtained supernatant after 2000 rpm centrifuging. Absorbance of supernatant was measured using UV-VIS spectrophotometer (Lambda 10, Perkin-Elmer, USA) at 412nm.

3.3.2.2. Liver tissue lipoperoxide content

Liver samples of propolis treated and non-treated animal (rat) were extracted, each sample (0.5g) was homogenized with its 9 times weight of 10 mM sodium phosphate buffer (pH 7.0). From this homogenized solution, 0.5 ml was taken, 3 ml mix of 1% phosphoric acid and 0.6% TBA was added to sample, heated for 45 min, n-butanol 4 ml was added and obtained supernatant after 2000 rpm centrifuging. Absorbance of supernatant was measured using UV-

VIS spectrophotometer (Perkin-Elmer, Lambda 10) at 535 nm.

3.3.2.3. Glutathione peroxidase activity (GSH-Px)

GSH-Px activity is determined by measuring absorbance decrease of glutathione reductase and NADH at 340 nm by oxidative glutathione, 1 unit of enzyme activity means amount of enzyme which generates 1 nmol of oxidative NADH during 1 min. Tris-HCl buffer (10 mM, pH 7.2) 2.6 ml was mixed with 30 mM reductive glutathione 0.1 ml and 6 ml NADPH solution (10 mM tris buffer NADPH 5 ul/ml) 0.1 ml, and reacted with 6.25 uM H₂O₂ for 5 min at 25°C. Sample 0.1 ml was added and reacted 5 min at 25°C, measure absorbance using UV-VIS spectrophotometer (Lambda 10, Perkin-Elmer, USA) at 340 nm.

3.3.2.4. Glutathione S-transferase activity (GST)

GST activity was determined by measuring absorbance of GSH-DNCB generated from reaction of 1-chloro-2,4-dinitrobenzene and glutathione using UV-VIS spectrophotometer (Lambda 10, Perkin-Elmer, USA) at 340nm.

3.4. Stomach cancer inhibitory effect of propolis

3.4.1. Identification of cell propagation ratio (MTT assay)

RAW 264.7 and SNU 484 cell lines from Korea Cell Line Bank were

cultivated using RPMI-1640 media mixed with 10% FBS (fetal bovine serum) at 37°C in CO₂ incubator. The cell lines were cultivated to 4×10⁵ cells/ml in 100ul media solution, their toxicity was identified at 96 well plate using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, USA) assay.

Cell lines treated with propolis were cultivated, MTT (5 mg/ml in saline) 10 ul was added each well and cultivated 90 min at 37°C, supernatant was removed, and cell lysis was done by adding 0.04N HCl 100ul. Absorbance was measured at 590nm by ELISA reader.

3.4.2 Identification of protein expression

Each entire cell samples dissolved for 1, 2, 3 day treatment were blocked with 5% fat-free milk, immunoblotted with primary antibody for 2 hours, HRP (horseradish peroxidase) secondary antibody (Chemicon, Single Oak Drive, Temecula, CA, USA) was added. The samples were dispersed using amplified chemiluminescent meter (Amersham Pharmacia, USA).

3.4.3. Investigation of stomach cancer inhibition effect of propolis (in vivo)

3.4.3.1. Cancer cell line and laboratory animal used for experiment

Cancer cell line used in this experiment was MKN 45 (human stomach

cancer) from Korean Cell Line Bank. Basic media was RPMI-1640 added with 10% FBS and penicillin-streptomycin, cultivation was conducted at 36.5°C in CO₂ incubator. The laboratory animal was nude mouse purchased from Orient Bio (Korea), 40 heads of 5 week old male nude mice were adapted for 1 week at Laboratory animal breeding center (Dept. of Veterinary Kangwon University) and used for experiment.

3.4.3.2. Observation of body weight alteration after cancer cell injection

Stomach cancer cell line (MKN 45) was injected into dorsal subcutaneous fat layer of each nude mice, these animals were grouped by normal (5 heads), control and treatment (low, medium, high concentration; each group 7 heads), treatment groups were allowed to drink propolis dissolved water (1.25 mg, 0.25 mg, 0.05 mg concentration) freely for 3 week after 24 hours from injection.

3.4.3.3. Observation of tumor size alteration after cancer cell injection

The tumor size (major and minor axis) of each mouse was measured by Digimatic Caliper (Mitutoyo, Japan), and mean volume of tumor was calculated.

$$\text{Mean tumor volume} = (\text{major axis}^2 \times \text{minor axis}^2)/2$$

3.4.3.4. Identification of COX-2 expression: RT-PCR

Each tumor tissue samples collected from control and treatment group were put into 5 ml tube, TRIzol agent 1 ml was added, tissue was dissolved by homogenize, and suspension was moved to sterilized eppendorf tube using pipette. Chloroform 100 ul was added, vortexing samples, centrifuged 15 min on the ice, supernatant was collected, reacted with 600 ul isopropanol for 20 min, and centrifuged for 15 min with 15,000 rpm at 4°C. Supernatant was removed, sediment was washed with 70% ethanol, centrifuged for 10 min with 15,000 rpm at 4°C, ethanol was removed, remained ethanol was vacuum dried, then sample was dissolved with DEPC treatment solution, and RNA concentration was measured using spectrophotometer.

PCR of separated RNA sample was conducted using cDNA Synthesis Kit. Separated RNA sample (1ug/ul concentration) 5 ul was denatured at 70°C for 10 min, PCR was done after mixing 5 × buffer 2 ul, dNTP 1 ul, DTT 0.25 ul, Oligo-dT 0.5 ul, RTase 0.3 ul, DEPC 0.95 ul, Total 10 ul. Reverse transcription was done for 10 min at 20°C, 60 min at 37°C, 5 min at 95°C, then kept at 4°C.

Reverse transcribed sample was mixed with sense primer 0.5 ul, antisense primer 0.5 ul, 10 × 5 ul buffer, dNTP 5 ul, MgCl₂ 6 ul, AmpliTag 0.3 ul and DEPC 22.7 ul, finally PCR was done with 50 ul of mixed sample. The PCR

condition and DNA sequences are as follows.

PCR cycle for COX-2

PCR cycle	25 cycles	
1. Denaturation	94°C	3 min
2. Annealing	94°C	30 sec
	58°C	30 sec
	72°C	5 min
3. Extension	72°C	10 min

COX-2 primer sequence

Sense	5'-TTAGCCTTGTGCACTGCAGA-3'
Antisense	5'-AGGAACAGCATGCAGGTAGC-3'

3.4.3.5. Statistical Analysis

One-way ANOVA was conducted using SPSS 18 program and corrected with Bonferroni test ($p < 0.05$).

IV. Results

1. Extraction characteristics of propolis by ethanol

1.1. Propolis yield of propolis by ethanol concentration

The extraction yield of propolis by ethanol concentration were identified (Figure 1), the extraction yield of Suwon propolis sample showed 10% until 40% EtOH, increased to over 30% with 60% EtOH and 44~46% over 70% EtOH without significant difference.

The extraction yield of Daegu sample showed over 35% at 60% EtOH, more than 40% over 70% EtOH. But yield of Jeju sample showed different tendency, which was less than 10% yield until 60% EtOH. The yield curve showed gradual increase at 10~20% yield section over 70% EtOH. The propolis yield value from Daegu and Suwon were more than 40% over 70% EtOH

But that of Jeju was more than 15% over 80% EtOH concentration. So the propolis yield of Jeju sample was lower than other samples from Daegu and Suwon.

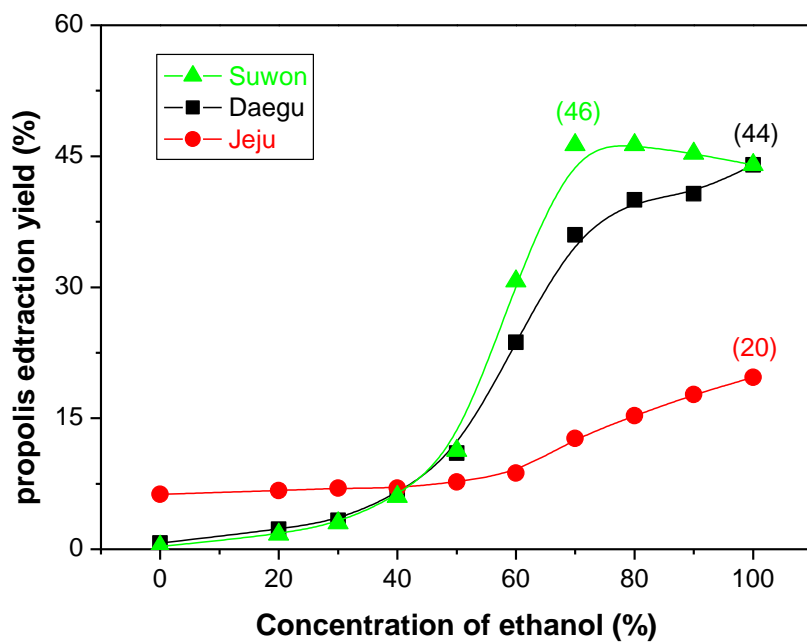


Figure 1. Extraction yield of propolis in accordance with the ethanol concentration for propolis extraction (25°C, 48hrs)

1.2. Propolis yield by extraction time

The yield of propolis by extraction time was examined (Figure 2), yield of Suwon sample was about 40% 1 hour after extraction, reached 60% after 6 hours, and showed 56% after 12 hours. The 56% yield was maintained without significant fluctuation after 24, 48 and 72 hours extraction.

Daegu sample showed 42% yield 1 hour after extraction, reached 50% after 12 hours, and showed little difference after extended extraction.

The yield of 1 hour extraction of Jeju sample was 15% and maintained about 20% after 12 hours. The yield of Jeju sample was lower than other samples but did not show significant difference in extraction time.

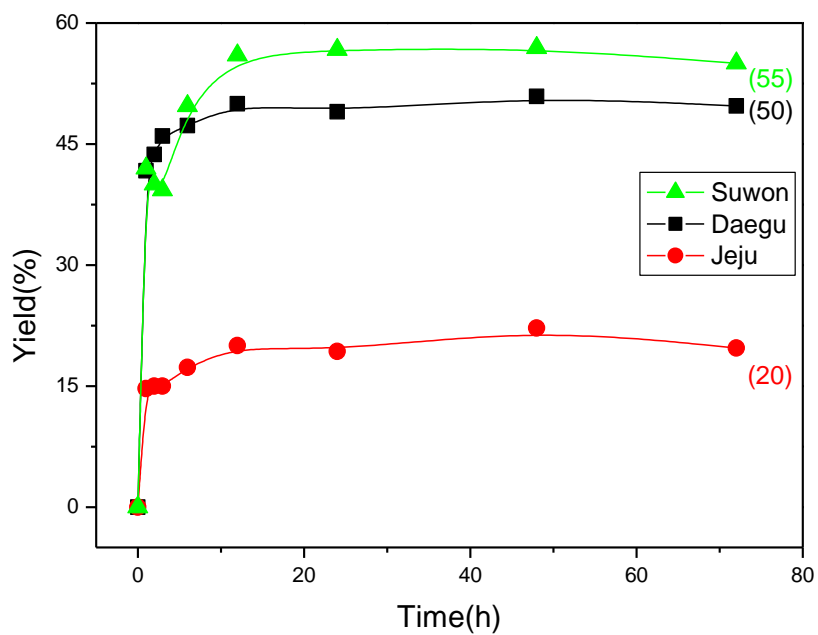


Figure 2. Extraction yield of propolis in accordance with the time (25°C, 80% EtOH) for propolis extraction.

1.3. Propolis yield by extraction temperature

The propolis yield of Suwon sample by extraction temperature was 45% at freezing condition (-20°C), 54% at cold condition (4°C), 57% at room temperature (25°C), and 57% at high temperature, respectively. The yield of Daegu sample did not show significant difference by temperature, it was 45% at freezing condition and 50% over the cold condition ($>4^{\circ}\text{C}$). The result of Jeju samples were 15% at freezing condition, 18% at cold condition (4°C), 21% at room temperature, and 27% at boiling temperature, the results showed that extraction yield increased to temperature rise (Figure 3).

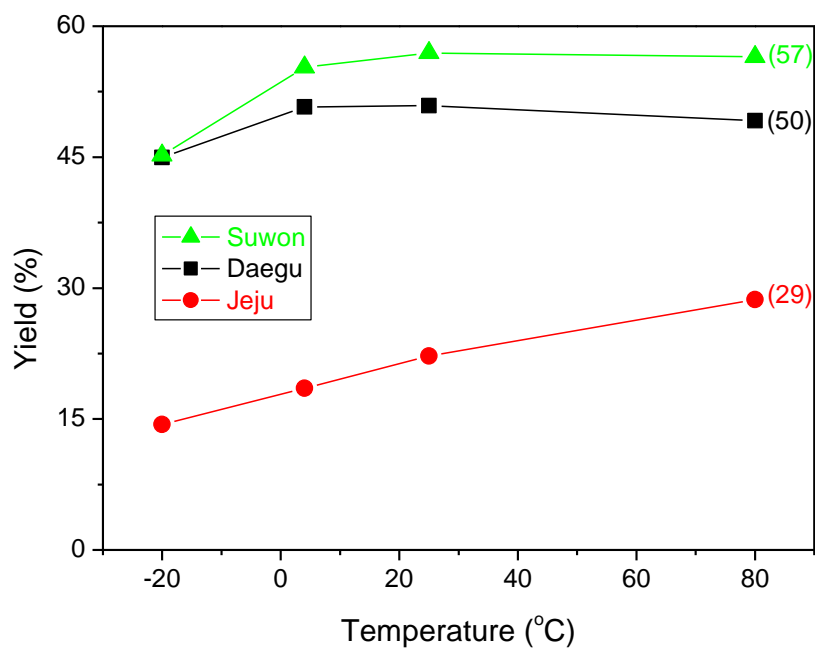


Figure 3. Extraction yield of propolis in accordance with extraction temperature (80% EtOH, 48hrs)

1.4. Propolis extraction by sonication

The extraction of propolis by sonication showed similar tendency of extraction by ethanol concentration (Figure 4).

Suwon sample showed 7% extraction yield at 40% EtOH, about 20% at 50% EtOH, and more than 46% over 60% EtOH. Propolis extraction yield from Daegu sample was about 15% at 40~50% EtOH, 35% at 60% EtOH, more than 40% over 70% EtOH. And Jeju sample's extraction yield showed higher value by increase of EtOH concentration.

The extraction yield of propolis by sonication was lower than that of ethanol extraction.

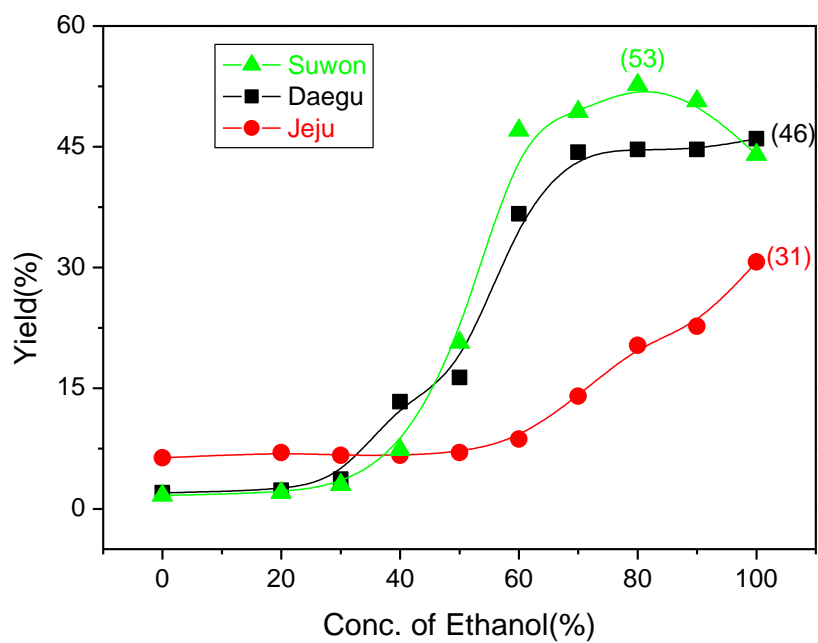


Figure 4. Extraction yield of propolis according to EtOH concentration by sonication for propolis extraction (25°C, 100min).

1.5. Comparison of total flavonoid contents by extraction method and ethanol concentration

Total flavonoid content of propolis extracted by EtOH concentration was determined (Table 1).

The total flavonoid content of Suwon sample was 3.54% by sonication with 40% EtOH, and 7~9% with little fluctuation over 60% EtOH. Propolis extracted at room temperature showed total flavonoid content of 4.62% with 40% EtOH, appeared to around 9% with more than 60% EtOH.

Sonicated Daegu sample showed higher (3.37~5.34%) content at 40~60% EtOH than room temperature extracted sample (2.50~4.12%), but it was lower (6.28~6.46) than room temperature treated sample (6.97~7.71) at 70~90% EtOH.

Most of Jeju sample showed very low content (less than 1%), sonication sample showed higher value than room temperature extracted sample. The less propolis extraction efficiency of sonication than room temperature extraction was confirmed by similar tendency of low total flavonoid content with lower yield by sonication comparing room temperature extraction.

Table 1. Total flavonoid contents (%) of propolis according to extraction method and EtOH concentration.

Region	Suwon		Daegu		Jeju	
Concentration of EtOH(%)	Sonication (%)	EtOH (%)	Sonication (%)	EtOH (%)	Sonication (%)	EtOH (%)
0 (water)	-	-	-	-	0.00	0.00
20	-	-	-	-	0.00	0.00
30	-	-	2.96	1.74	0.02	0.00
40	3.54	4.62	3.37	2.50	0.05	0.11
50	7.50	8.16	5.14	4.04	0.21	0.11
60	8.68	9.35	5.34	4.12	0.15	0.11
70	6.87	9.36	6.44	6.94	0.24	0.18
80	8.75	9.94	6.28	7.02	0.34	0.00
90	8.39	9.27	6.46	7.71	0.29	0.00
99.9 (pure EtOH)	7.75	8.61	6.25	5.70	0.14	0.00

1.6. Comparison of total contents of flavonoid and phenolic by ethanol concentration

The total flavonoid content of Suwon & Daegu propolis samples by EtOH concentration was about 3% under 20% EtOH, then increased from 40% EtOH and showed highest value at 70% EtOH. Jeju sample showed almost zero total flavonoid content.

The extraction yield and total flavonoid content were excellent at 70% ethanol (When used as food is edible alcohol use). And this was consistent with the result (Kujumgiev *et al.*, 1999; Santos *et al.*, 2002; Lu *et al.*, 2005; Mani *et al.*, 2006) which used 70~100% ethanol for propolis extraction.

On the contrary, total phenolic content of Suwon & Daegu samples showed highest content (24, 20g/100g gallic acid) at 50% EtOH and decreased by EtOH concentration rise. Jeju sample showed highest content (12g/100g gallic acid) at 60% EtOH, and decreased by EtOH concentration rise (Figure 5).

Propolis yield and total flavonoid content had tendency to increase by EtOH concentration rise until 70%, but total phenolic content was highest at 50~60% EtOH, and decreased by EtOH concentration rise.

The total phenolic content of propolis extract was highest when 50 to 60% ethanol, 50% ethanol, 24% in Suwon, Daegu in 50% ethanol, 20%, Jeju total phenolic content of 12% in 60% ethanol are shown.

This suggests that propolis extraction is more efficient than just using alcohol spirit when proper amount of water is mixed with ethanol. Twelve hour was enough for full extraction of propolis, and extraction temperature was no matter except freezing condition.

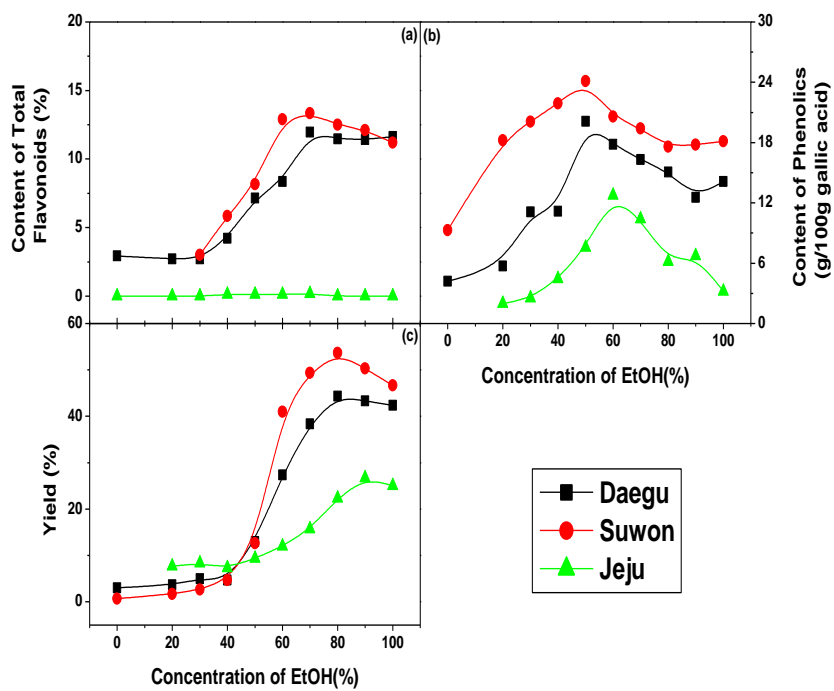


Figure 5. Total flavonoid (a), total phenolic (b) and yield (c) according to EtOH concentration

2. General characteristics of propolis collected by time and origin (Korean and foreign)

2.1. Total flavonoid and phenolic content of propolis by collection time

2.1.1. Propolis collection amount by time

The amount of collected propolis and ingredient composition by time were analyzed. The collected amount of propolis in 2003 was about 47g from early June to mid-July, 40g during August, and total 100g through 2003 (Figure 6). In 2004, collection amount was about 30g in June, about 48g from the middle of July to the end of August, and the collection amount decreased rapidly after the end of August (Figure 7).

This may need to be removed from the hive to collect network or plate before and after Thanksgiving (Chuseok) when you collect propolis, stop before October the propolis collected, it is preferable to have no effect on the winter beehive.

2.1.2. Total flavonoid and phenolic content of propolis collected by time

Total flavonoid content of propolis collected by time was 7~8%, showed no significant difference among collection time, and total phenolic content was

16~20% range (Figure 8). When compared with foreign data, Korean propolis showed similar values (Teixeira *et al.*, 2010).

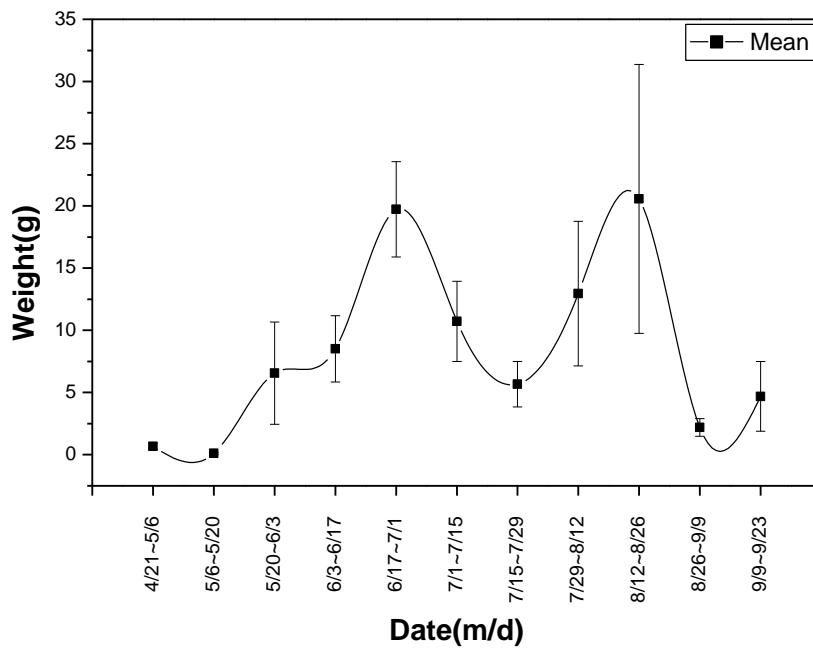


Figure 6. Weights of propolis collected every two weeks in 2003. Error bar is standard errors.

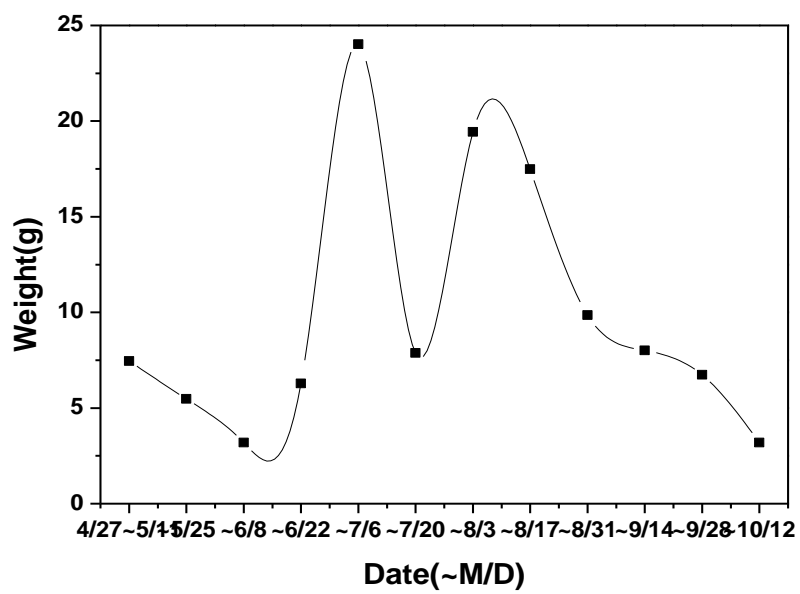


Figure 7. Weights of propolis collected every two weeks in 2004.

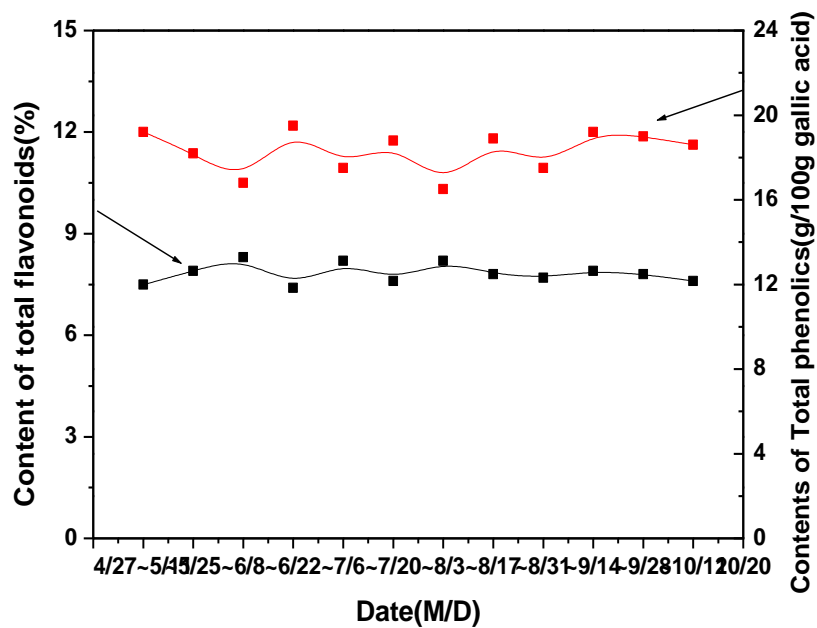


Figure 8. Total flavonoid and total phenolic contents of propolis collected every two weeks

2.1.3. HPLC analysis of propolis collected by time

The HPLC analysis result of propolis collected by time showed different peak height, in particular, showed a lot of the difference at 28 minutes and 42 minutes, depending on the season (Figure 9).

These results demonstrate that propolis is one component that varies depending on when the collection because it comes from plants having around the beehive.

The component change by the collection time was identified from HPLC analysis which showed the different peak height and position (Simões-Ambrosio *et al.*, 2010).

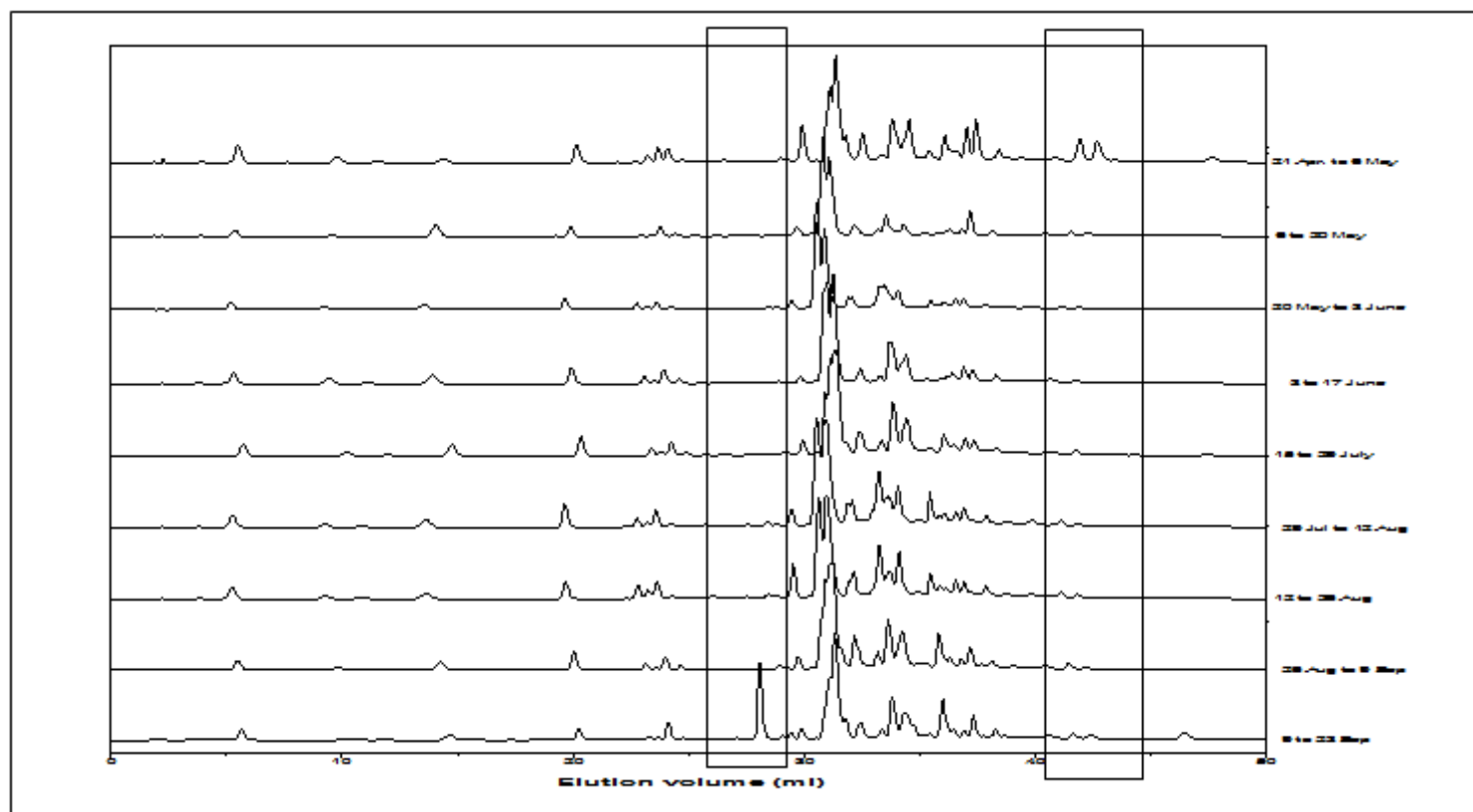


Figure 9. HPLC chromatogram of propolis collected every two week

2.2. Ingredient analysis of propolis

2.2.1. Total flavonoid & phenolic content of propolis

The grade of propolis is based on the total flavonoid content from Health Functional Food Act, total flavonoid content for analysis of the collected propolis was measured.

Propolis collected in most areas showed a total flavonoid content of 4-8%.

This means functional health food revolution in the raw material itself was the level at which one can take full advantage of propolis as raw material meets the present at least 5%.

Propolis collected in Jeju showed a value less than 1% total flavonoid content (Figure 10).

That the high content of phenolic compounds in the known propolis total phenolic content was measured to confirm that the content of polyphenols.

In order to confirm the content of the polyphenols that are known in the propolis content high, showed a value of at least 20% measured as a result most of the total phenolic content, Jeju exhibited the mean value of 15%

The inland propolis sample showed more than 20% total phenolic content (Kumazawa *et al*, 2004; Bonvehí & Gutiérrez, 2012) and Jeju sample showed about 15% content (Figure 11).

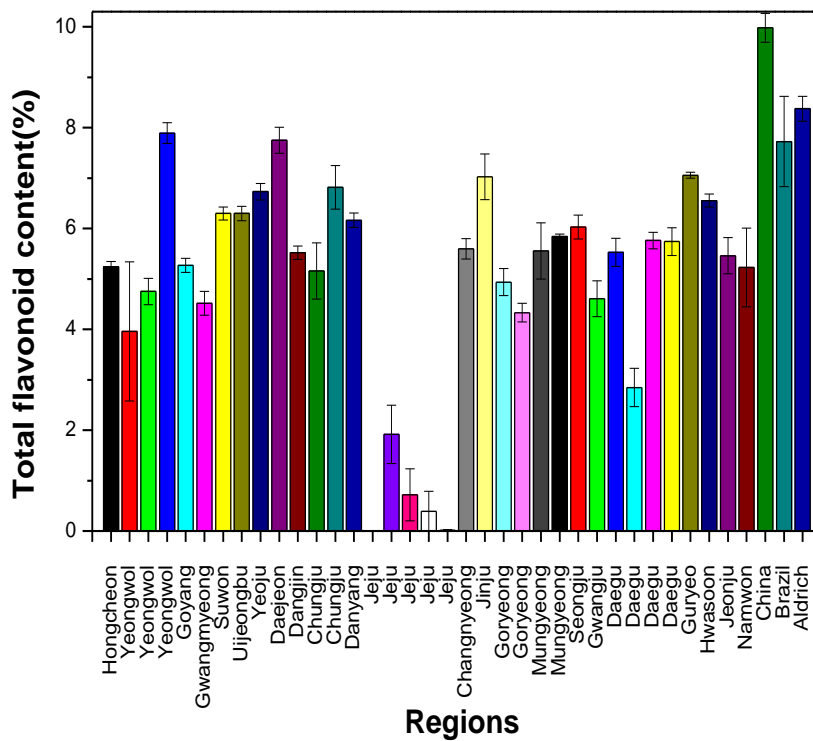


Figure 10. Total flavonoid contents of propolis by region. Error bars are standard errors

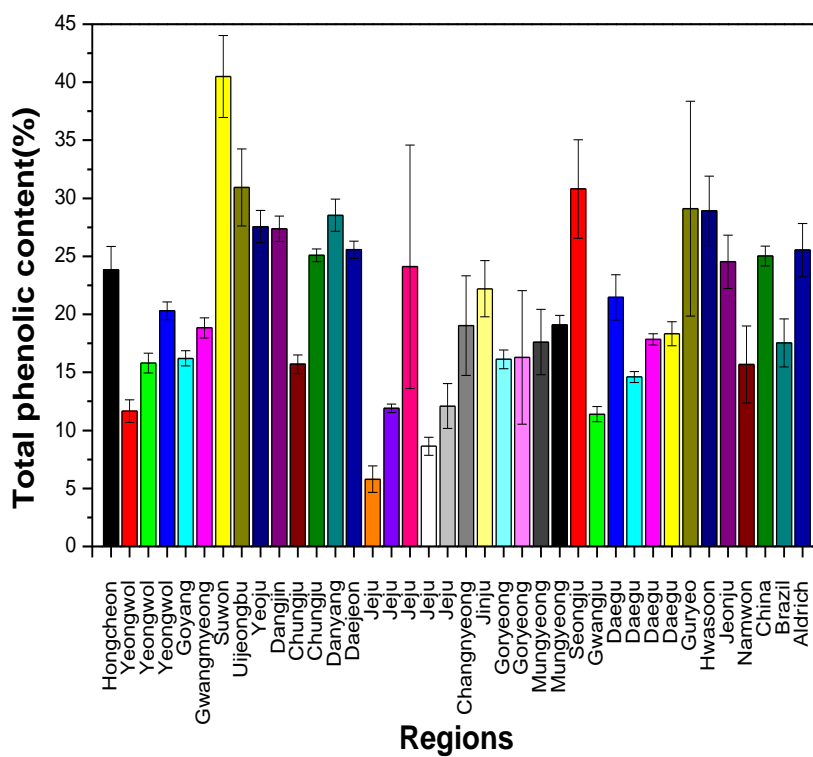


Figure 11. Total phenolic contents of propolis by region.

Error bars are standard errors.

2.2.2. Identification of amino acid content in propolis

Propolis was contained trace amounts of 17 kinds of amino acids (Table 2), all samples contained Aspartic acid (Asp), Serin (Ser), Glycine (Gly), Methionine (Met), most of samples except 1~2 regions contained Glutamic acid (Glu), Valine (Val), Cysteine (Cys), Proline (Pro), and less than one-third of samples contained Threonine (Thr), Phenylalanine (Phe), Histidine (His).

Jeju Propolis has been found to be a little much higher content than any sort of inland propolis.

Amino acids contained in the propolis was primarily known to be derived from the pollen.

Table 2. Amino acid contents of propolis

(unit: nmol)

	Asp	Thr	Ser	Glu	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	Pro
Hongcheon	013	-	039	008	004	006	011	020	014	006	005	008	-	-	002	-	022
Yeongwol	027	-	196	020	648	448	016	045	016	014	014	019	017	006	029	042	011
Yeongwol	009	006	005	007	006	-	011	019	019	-	006	002	-	-	-	-	018
Yeongwol	011	-	012	006	007	-	012	017	019	-	004	-	-	004	016	005	013
Goyang	008	-	012	002	018	013	012	016	024	-	-	008	-	-	-	-	002
Gwangmyeong	005	-	280	021	862	632	-	006	014	016	014	012	021	026	009	005	018
Suwon	010	-	024	008	006	008	011	019	019	-	005	008	-	-	-	024	008
Uijeongbu	008	-	016	005	029	018	011	017	018	-	005	-	-	-	-	-	021
Yeoju	013	-	014	007	008	002	011	017	015	006	006	002	-	-	-	-	011
Dangjin	009	004	004	008	008	-	011	019	019	-	006	-	-	000	014	007	005
Daejeon	011	-	016	008	024	017	011	016	023	-	-	002	-	-	-	004	012
Chungju	014	008	014	017	014	009	011	019	014	008	006	004	-	010	008	021	101
Chungju	010	005	005	004	006	008	011	017	015	005	006	-	-	-	002	008	012
Danyang	012	007	009	011	014	006	011	020	015	006	007	008	-	012	008	027	009
Jeju	013	006	008	007	013	008	011	019	014	007	007	002	-	-	002	-	006
Jeju	031	021	085	034	215	162	-	064	015	018	018	009	013	011	005	006	050
Jeju	014	006	010	007	011	008	011	019	014	-	015	-	006	-	002	004	012
Jeju	017	008	011	017	013	006	011	021	014	008	010	008	-	-	005	024	037
Jeju	019	013	020	039	025	015	029	-	014	010	011	008	008	017	006	008	006
Changnyeong	013	-	045	011	115	009	012	023	016	007	010	007	-	005	029	037	003
Jinju	010	-	011	008	010	007	011	018	024	-	-	-	-	-	-	-	008
Goryeong	010	-	010	007	006	008	011	018	018	-	005	-	-	-	-	013	046
Goryeong	010	-	007	008	008	-	011	018	023	-	-	-	-	-	-	025	007
Mungyeong	008	-	006	002	002	-	014	019	034	-	-	006	-	005	027	030	009
Mungyeong	008	044	004	007	006	008	011	019	016	005	006	002	-	-	004	024	051
Seongju	012	006	005	006	007	008	011	018	013	006	006	002	-	-	002	-	027
Gwangju	008	-	021	005	041	027	011	017	021	-	-	-	-	-	-	-	027
Daegu	008	-	008	-	008	-	013	018	022	-	005	-	-	000	007	-	006
Daegu	008	005	004	009	006	004	012	018	021	-	007	006	-	007	027	027	024
Daegu	002	-	007	004	006	002	011	017	018	-	006	-	-	-	002	002	007
Daegu	014	-	021	010	005	020	011	019	013	007	006	004	-	-	004	025	028
Guryeo	012	-	047	005	137	006	012	021	013	006	006	004	-	-	-	-	008
Hwasoon	009	-	020	002	051	005	011	017	019	-	005	008	-	-	-	-	008
Jeonju	002	-	005	029	004	-	011	017	020	005	-	-	-	-	-	-	017
Namwon	007	-	017	004	028	018	011	017	018	-	005	002	-	-	-	-	004
China	009	-	007	002	008	-	012	016	020	-	004	-	-	016	-	-	-
Brazil	024	012	015	051	019	019	011	023	014	008	010	008	-	023	006	007	012
Aldrich	010	-	009	005	005	002	011	018	014	005	006	-	-	-	-	005	006

2.2.3. Color of propolis

Results confirm the color of the propolis collected from each region, showed a range of colors from yellow to brown, propolis is collected in Jeju lighter colors similar to what was shown to yield less than those in other regions. But other regions samples in Korea did not show indigenous color by region (Table 3).

Propolis is collected from each region, and represents a unique color, dependent on the tree is that any collection area in color. Was observed that propolis collected in Jeju yield also appears lighter colors similar to what appeared to be less than in other regions, outside of Jeju is not intended to represent a certain color depending on the region (Burdock, 1997).

Table 3. Colors of propolis on each region

Region		HVC
Cetral	Hongcheon	6.94RP 2.11 / 0.30
	Yeongwol	0.71YR 2.15 / 0.24
	Yeongwol	0.88YR 2.20 / 0.31
	Yeongwol	8.05RP 2.07 / 0.38
	Goyang	8.28R 2.15 / 0.28
	Gwangmyeong	5.85RP 2.11 / 0.28
	Suwon	6.44R 2.16 / 0.43
	Uijeongbu	9.69RP 2.08 / 0.38
	Yeoju	3.66YR 2.18 / 0.39
	Dangjin	9.15RP 2.09 / 0.35
	Chungju	5.35YR 3.18 / 0.55
	Chungju	7.41R 2.14 / 0.28
	Danyang	8.96R 2.18 / 0.41
	Daejeon	8.14R 2.15 / 0.38
Southern	Changnyeong	9.06R 2.20 / 0.45
	Jinju	9.49R 2.17 / 0.35
	Goryeong	6.86RP 2.08 / 0.44
	Goryeong	8.82RP 2.09 / 0.39
	Mungyeong	8.08RP 2.11 / 0.21
	Mungyeong	7.40R 2.15 / 0.27
	Seongju	7.12R 2.15 / 0.41
	Gwangju	6.12R 2.17 / 0.25
	Daegu	2.97RP 2.09 / 0.41
	Daegu	4.23RP 2.10 / 0.50
	Daegu	9.64RP 2.10 / 0.39
	Daegu	7.82RP 2.08 / 0.38
	Guryeo	2.24R 2.12 / 0.31
	Hwasoon	5.30RP 2.07 / 0.33
	Jeonju	5.63YR 2.19 / 0.38
Jeu	Namwon	3.66RP 2.13 / 0.24
	Jeju	2.01Y 2.25 / 0.22
	Jeju	4.72Y 2.26 / 0.14
	Jeju	8.31R 2.15 / 0.24
	Jeju	6.94Y 2.25 / 0.33
abroad	Jeju	2.55Y 2.22 / 0.31
	China	5.50PB 1.93 / 0.57
	Brazil	5.14RP 2.27 / 0.09
	Aldrich	5.02PB 1.94 / 0.63

※ HVC: Hue, Value, Chroma

※ R: red, RP: red purple, Y: Yellow, YR: Yellow Red, PB: purple blue

2.2.4. Heavy metal contents of propolis

Analysis of heavy metal content revealed less than standard (Health Functional Food Act, less than 5ppm) content of lead (Pb) and negative detection of arsenic (As) and mercury (Hg). Slight amount of minerals (Cu, Zn) necessary for metabolism (Ana Haro *et al.*, 2000) was detected (Table 4).

2.2.5. Crude lipid contents of propolis

The crude lipids of propolis is wax and fatty acid originated from beeswax and plants, and its content is known as 25~40%. The crude lipid content of propolis samples is average 37.0% from central region, about 39.1% from Southern region, 23.8% from Jeju. There is some difference between inland and island (figure 12).

Table 4. Heavy metal and mineral contents of propolis (unit: ppm)

Region		Cd	Cr	Pb	As	Hg	Cu	Ni	Zn
Central	Hongcheon	nd	nd	nd	nd	nd	nd	nd	0.230
	Yeongwol	nd	nd	nd	nd	nd	0.035	nd	0.610
	Yeongwol	nd	nd	nd	nd	nd	0.048	0.003	0.323
	Yeongwol	nd	nd	nd	nd	nd	nd	nd	0.271
	Goyang	nd	nd	nd	nd	nd	nd	nd	0.231
	Gwangju	nd	nd	nd	nd	nd	0.007	0.013	0.430
	Suwon	nd	nd	nd	nd	nd	nd	nd	0.301
	Uijeongbu	nd	nd	nd	nd	nd	nd	0.001	0.387
	Yeoju	0.008	nd	0.184	nd	nd	nd	0.019	0.315
	Dangjin	nd	nd	nd	nd	nd	nd	nd	0.256
South	Chungju	nd	nd	nd	nd	nd	0.071	0.008	0.270
	Chungju	nd	nd	nd	nd	nd	nd	nd	0.848
	Danyang	nd	nd	nd	nd	nd	nd	nd	0.354
	Daejeon	nd	nd	nd	nd	nd	nd	nd	0.265
	Changnyeong	nd	nd	nd	nd	nd	nd	nd	0.280
	Jinju	nd	nd	0.070	nd	nd	0.016	0.002	0.449
	Goryeong	nd	nd	nd	nd	nd	nd	nd	0.229
	Goryeong	nd	nd	0.051	nd	nd	nd	0.002	0.364
	Mungyeong	nd	nd	nd	nd	nd	0.040	0.006	0.246
	Mungyeong	nd	nd	nd	nd	nd	nd	nd	0.445
Jeju	Seongju	nd	nd	nd	nd	nd	0.002	0.003	0.338
	Gwangju	nd	nd	nd	nd	nd	nd	nd	0.268
	Daegu	nd	nd	nd	nd	nd	0.012	nd	0.259
	Daegu	nd	nd	nd	nd	nd	0.001	0.067	0.672
	Daegu	nd	nd	nd	nd	nd	0.027	0.003	0.282
	Daegu	nd	nd	nd	nd	nd	nd	0.007	0.314
	Guryeo	nd	nd	0.017	nd	nd	0.053	nd	0.515
	Hwasoon	nd	nd	nd	nd	nd	nd	nd	0.242
	Jeonju	nd	nd	nd	nd	nd	nd	0.001	0.229
	Namwon	nd	nd	0.089	nd	nd	0.130	0.004	0.579
abroad	Jeju	0.017	nd	0.004	nd	nd	0.031	0.006	0.258
	Jeju	nd	nd	nd	nd	nd	0.014	0.001	0.472
	Jeju	nd	nd	nd	nd	nd	0.016	0.028	0.507
	Jeju	0.018	nd	0.538	nd	nd	0.021	0.013	0.600
	Jeju	0.008	nd	nd	nd	nd	0.031	0.002	0.651
abroad	China	nd	nd	0.192	nd	nd	0.032	0.012	0.739
	Brazil	nd	nd	0.046	nd	nd	0.111	0.130	0.507
	Aldrich	nd	0.497	18.21	0.014	nd	0.119	0.036	3.132

※ nd : not detected

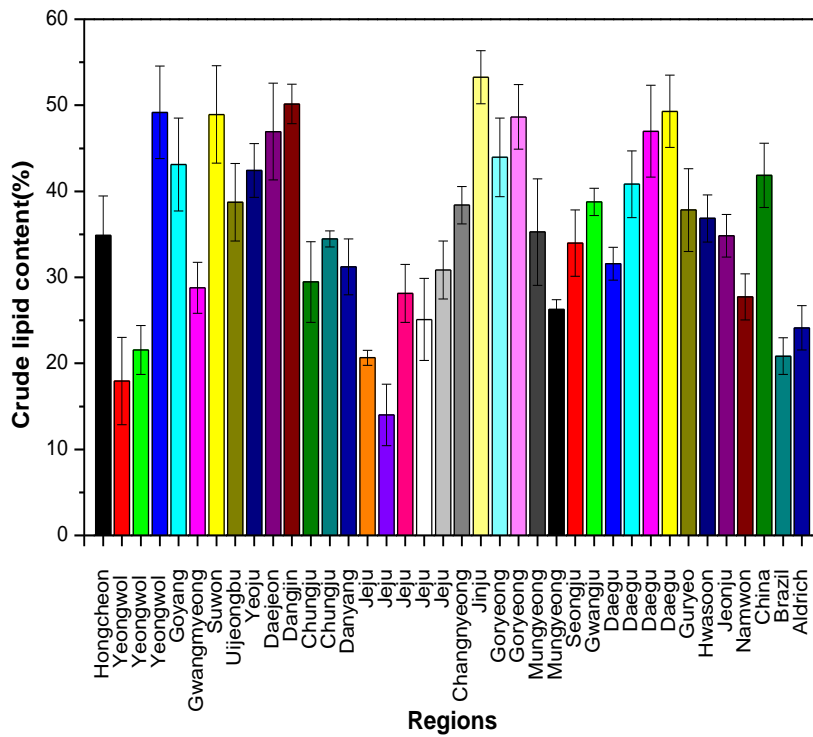


Figure 12. Crude lipid contents of propolis by collected region. Error bars are standard errors.

2.3. HPLC analysis of flavonoids in Korean propolis

For the analysis of flavonoids contained in Korean propolis, HPLC results were compared to standards (Figure 13). Each peak of standards are a: Gallic acid (2min), b: Naringenin (23min), c: Quercetin (27min), d: Apigenin (31min), e: Chrysin (34min), and f: Galangin (37min).

The Jeju 1 propolis sample was negative of naringenin and quercetin, and showed other peaks at 47min and 51 min. All of six peaks were identified with Jeju 2 sample (Figure 14).

Central region samples from Chungju and Goyang showed all 6 peaks, and Yeongwol sample showed all 6 main peaks and two more peaks at 42 min and 45 min (Figure 15).

Southern region sample from Daegu was negative to naringenin, Hwasun sample did not have quercetin peak (Figure 16).

The Chinese sample did not have gallic acid peak, showed new peaks at 32min, 35 min and 43 min. Brazilian propolis showed quercetin and galangin, but no other peaks were identified, and its indigenous peaks were identified at 39 min and 44 min (Figure 17).

These different peaks suggests reflection of different woody and herbal plant species characteristics of originated region (Alencar *et al.*, 2007; Guo *et al.*, 2011; Kumazawa *et al.*, 2013).

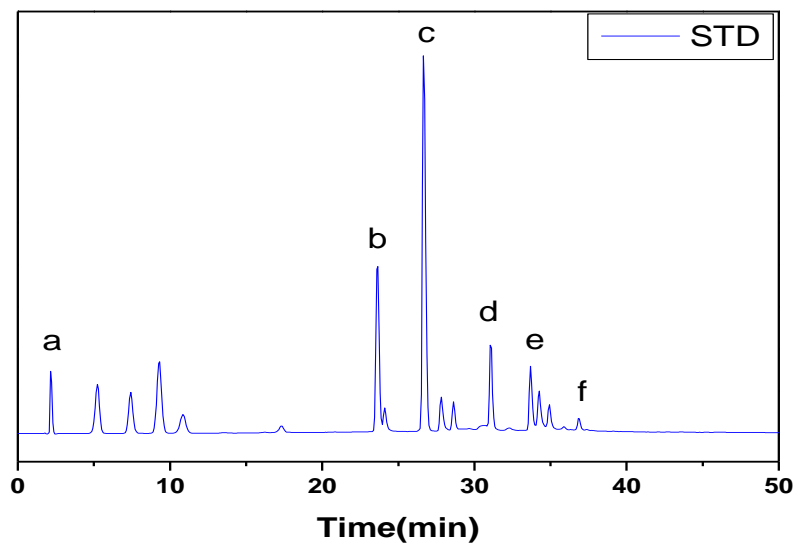


Figure 13. HPLC Chromatogram of standards (a: Gallic acid, b: Naringenin, c: Quercetin, d: Apigenin, e: Chrysin, f: Galangin)

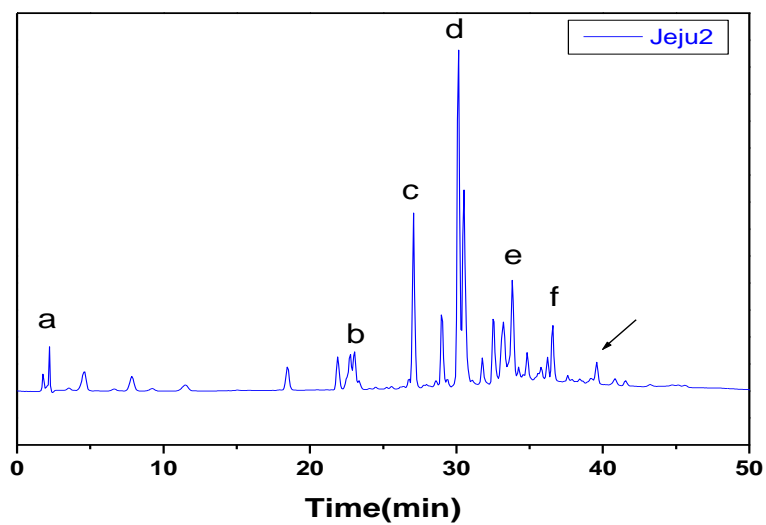
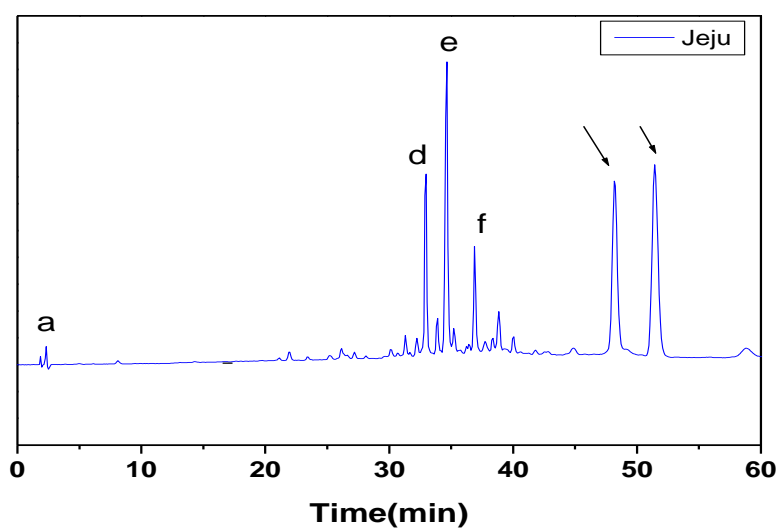


Figure 14. HPLC Chromatogram of propolis collected from Jeju island (a: Gallic acid, b: Naringenin, c: Quercetin, d: Apigenin, e: Chrysin, f: Galangin)

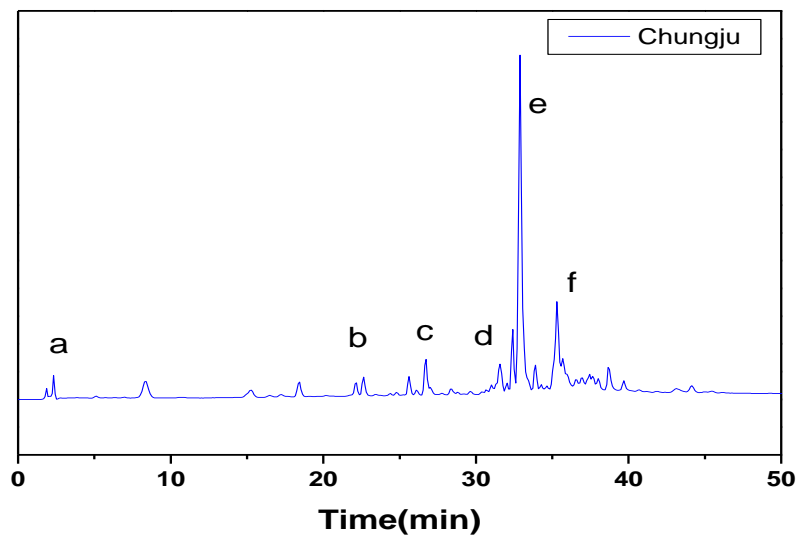
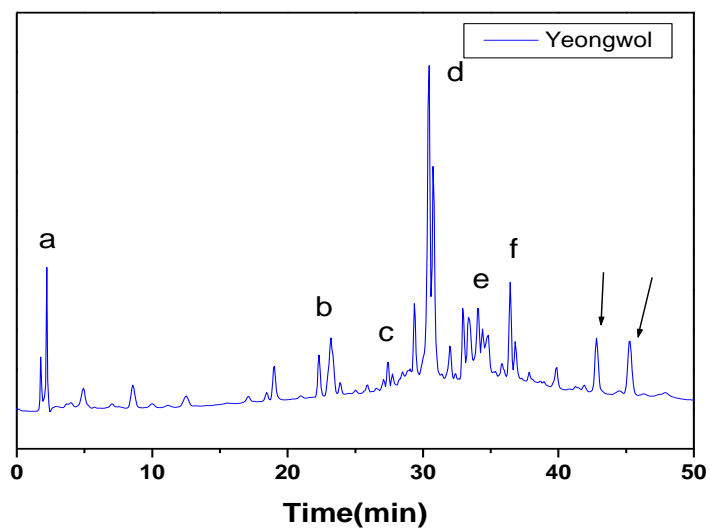
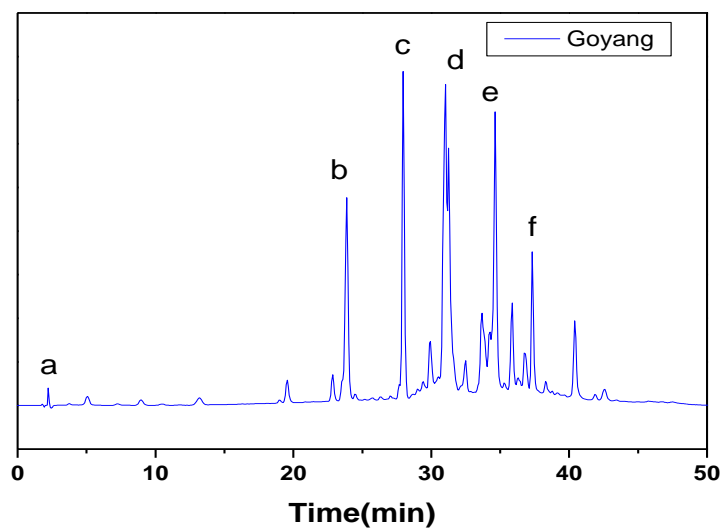


Figure 15. HPLC Chromatogram of propolis collected from central region (a: Gallic acid, b: Naringenin, c: Quercetin, d: Apigenin, e: Chrysin, f: Galangin)



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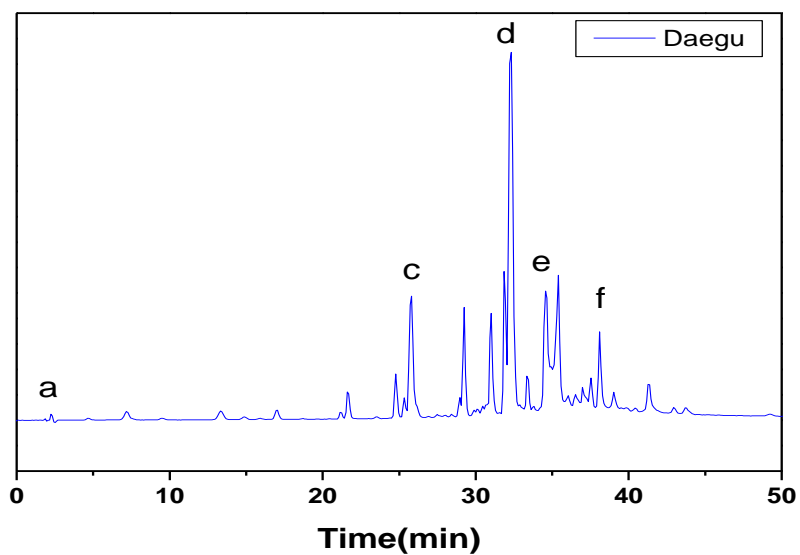
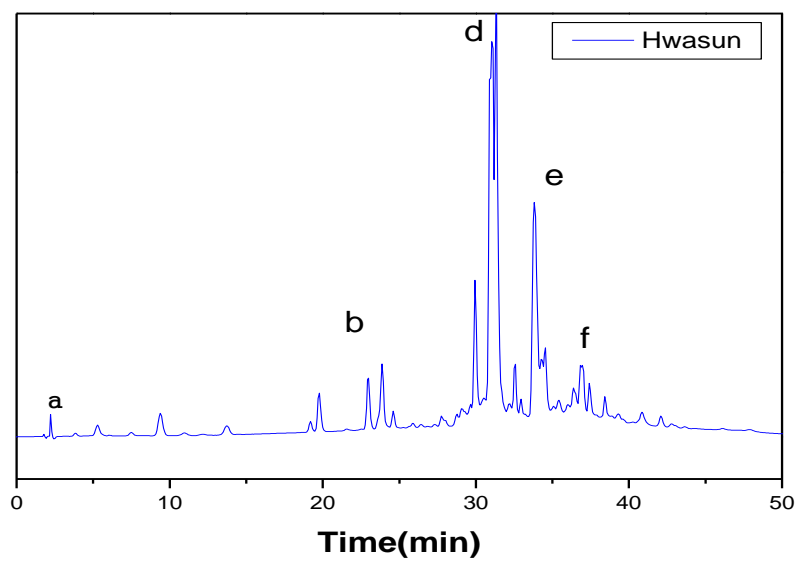
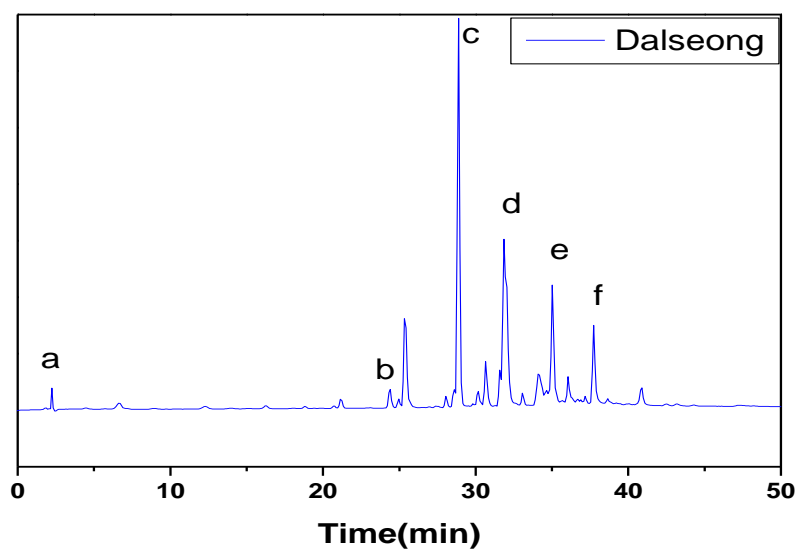


Figure 16. HPLC Chromatogram of propolis collected from southern region
(a: Gallic acid, b: Naringenin, c: Quercetin, d: Apigenin, e:
Chrysin, f: Galangin)



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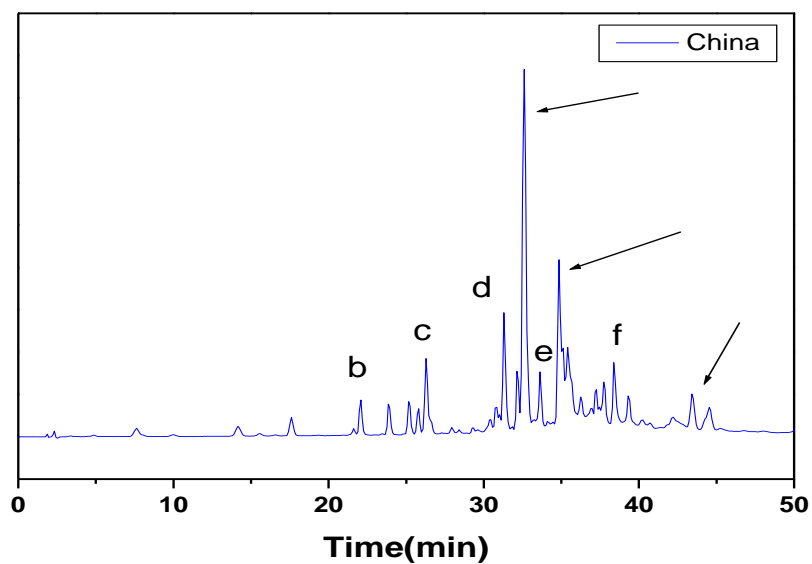


Figure 17. HPLC Chromatogram of propolis collected from China (a: Gallic acid, b: Naringenin, c: Quercetin, d: Apigenin, e: Chrysin, f: Galangin)

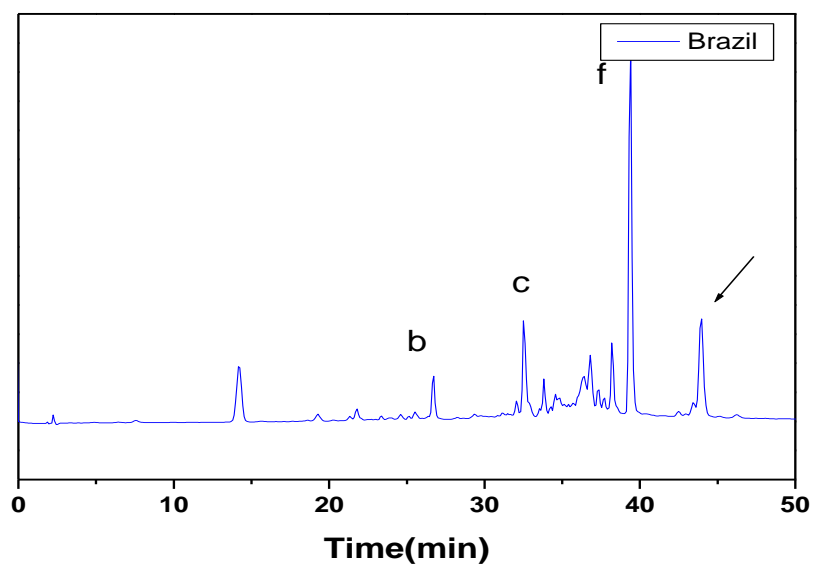


Figure 18. HPLC Chromatogram of propolis collected from Brazil (a: Gallic acid, b: Naringenin, c: Quercetin, d: Apigenin, e: Chrysin, f: Galangin)

Table 5. Flavonoid and phenolic contents of HPLC chromatogram on regional propolis.

	a	b	c	d	e	f
	Gallic acid	Naringenin	Quercetin	Apigenin	Chrysin	Galangin
STD	444	1946	4364	895	568	166
Central						
Chungju	31.2	52	104	107	786	272
Goyang	23	752	1005	1341	444	245
Yeongwol	122	181	108	513	253	259
Southern						
Daegu	10	-	263	1257	404	206
Dalseong	46	100	1346	1030	510	359
Hwasun	25	225	-	1436	912	309
Jeju						
1	6.7	-	-	169	280	91
2	103	200	780	1019	632	284

2.4. Investigation and identification of propolis index material

2.4.1. Separation of propolis by prepHPLC

Propolis was fractioned by prepHPLC using JAIGEL-GS310 column, the range was set up by checking with UV detector, and divided into 21 of 2 min fractions with 3ml/min dispersion speed (Figure 19).

2.4.2. HPLC analysis of separated propolis

The 21 fractions divided by prepHPLC were examined by HPLC (Figure 20). Various peaks were observed by each fraction, and the peaks became simpler to the end of fractions.

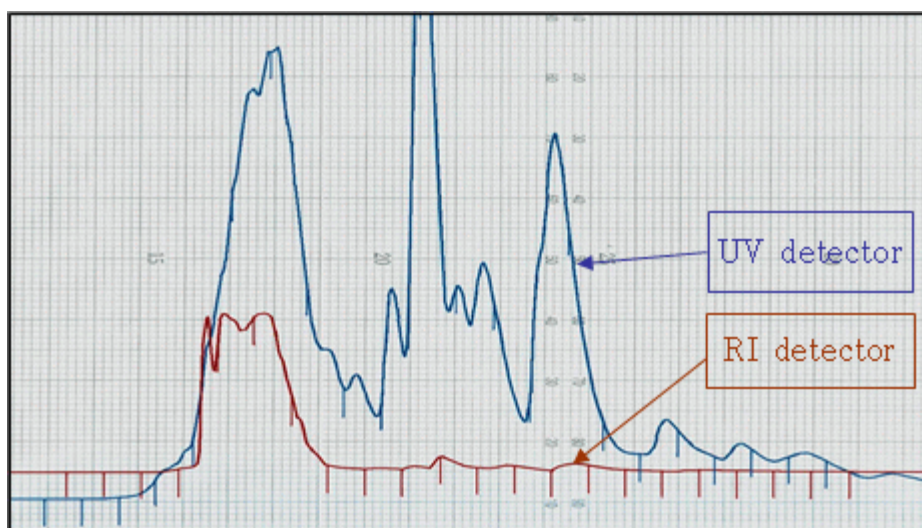


Figure 19. prepHPLC graph of 21 fractions with propolis

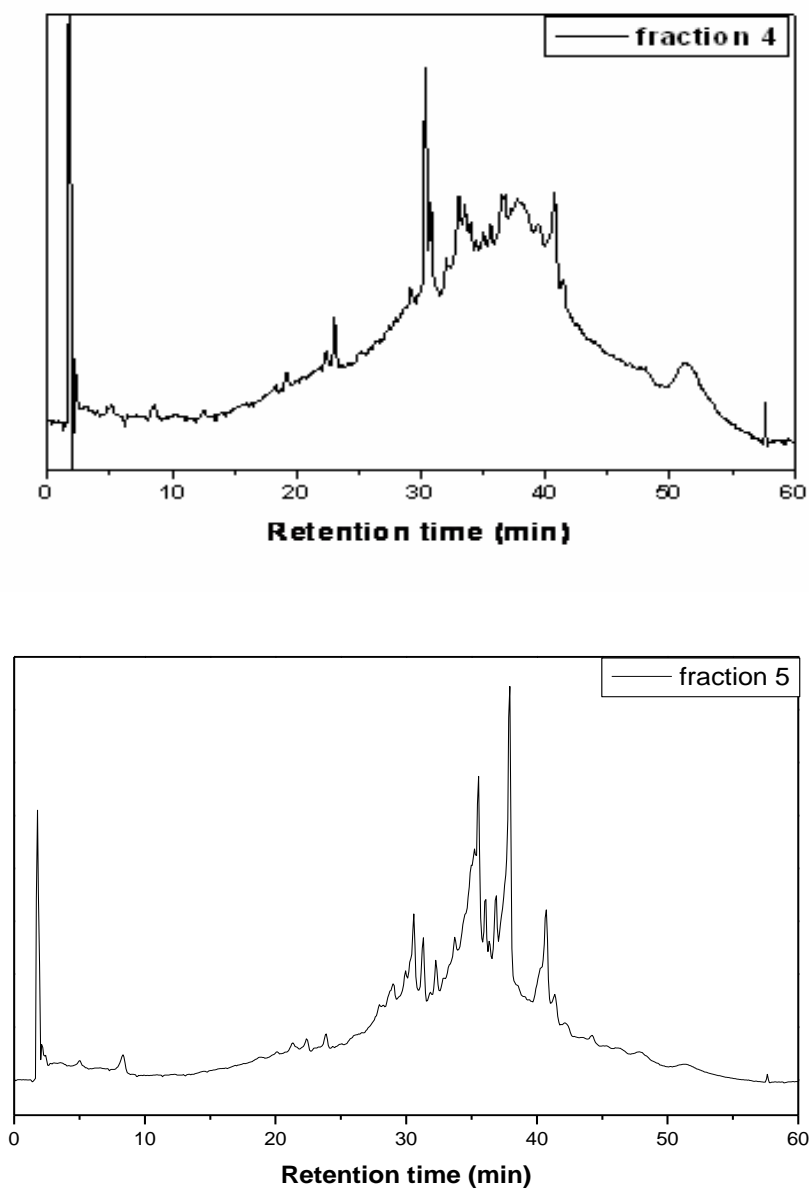
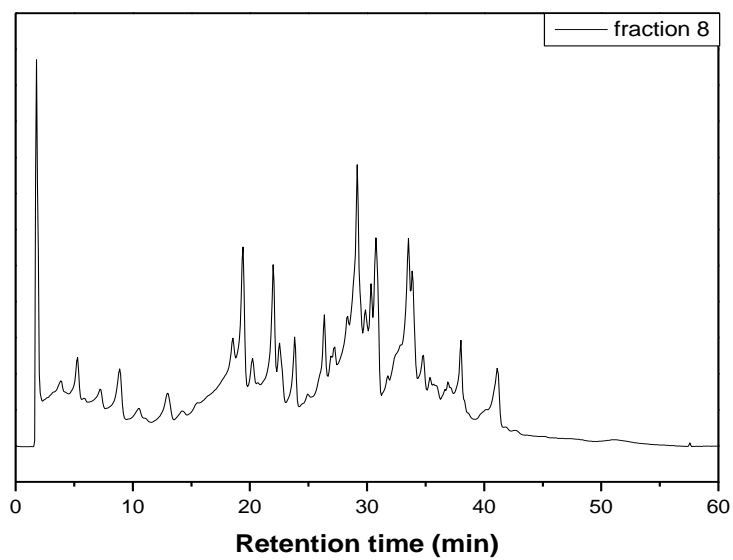
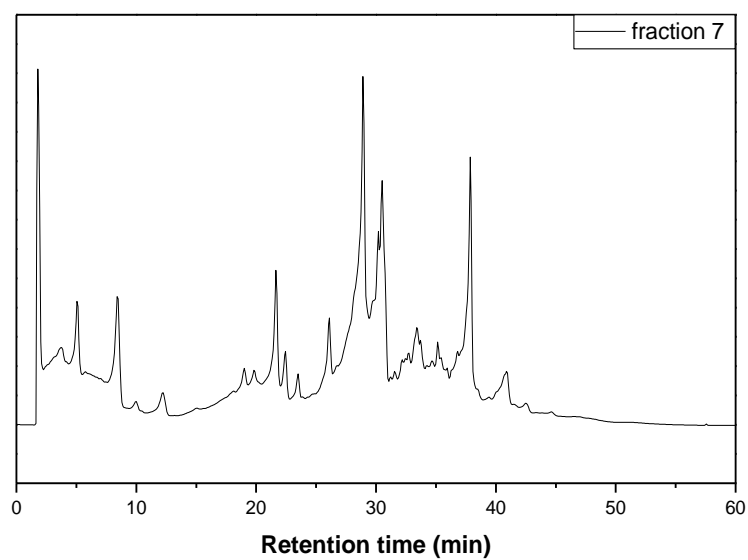
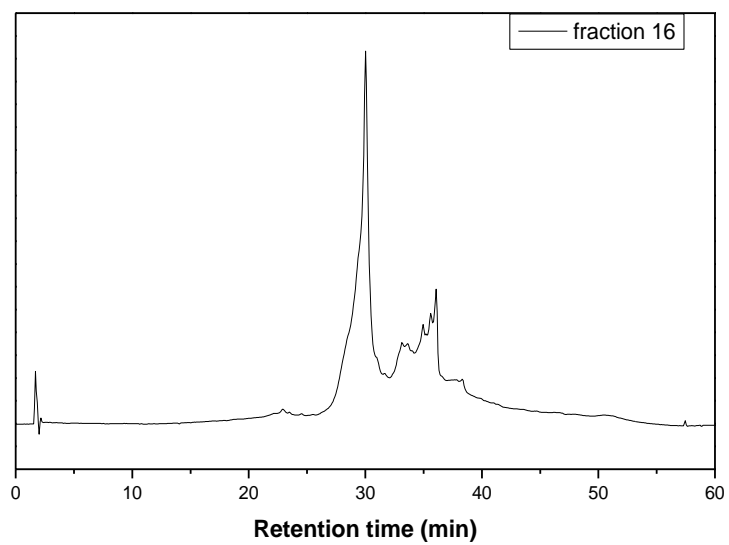
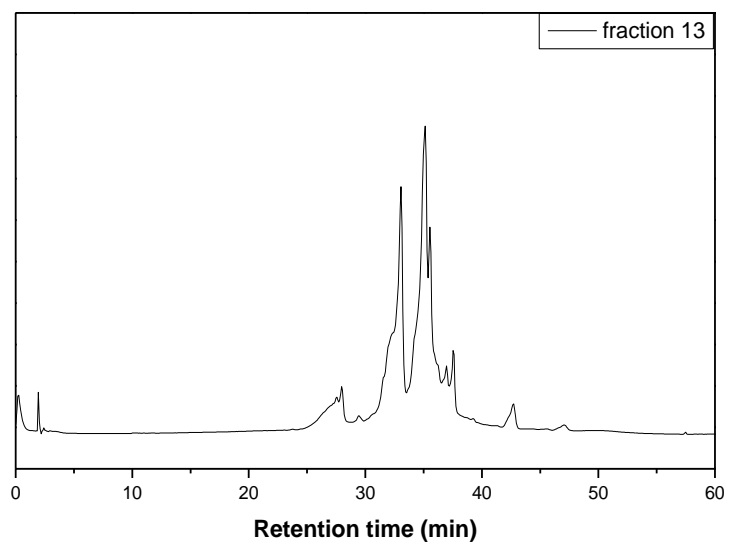


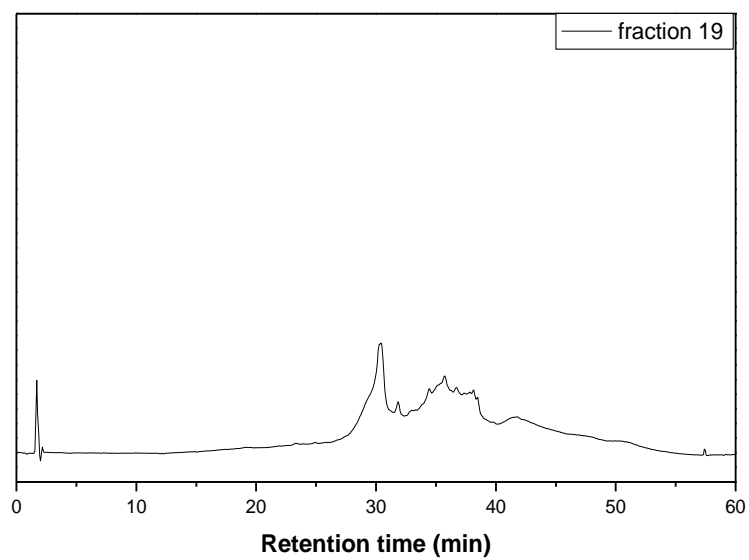
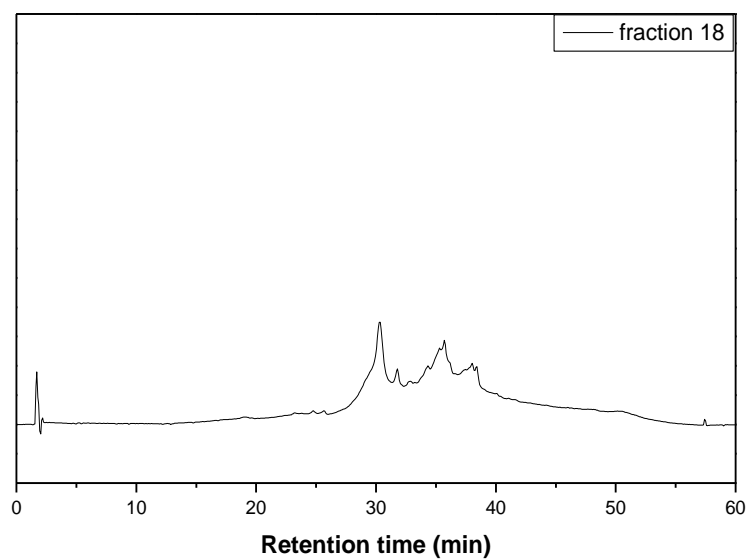
Figure 20. HPLC chromatogram of the fractions of propolis separated by prepHPLC



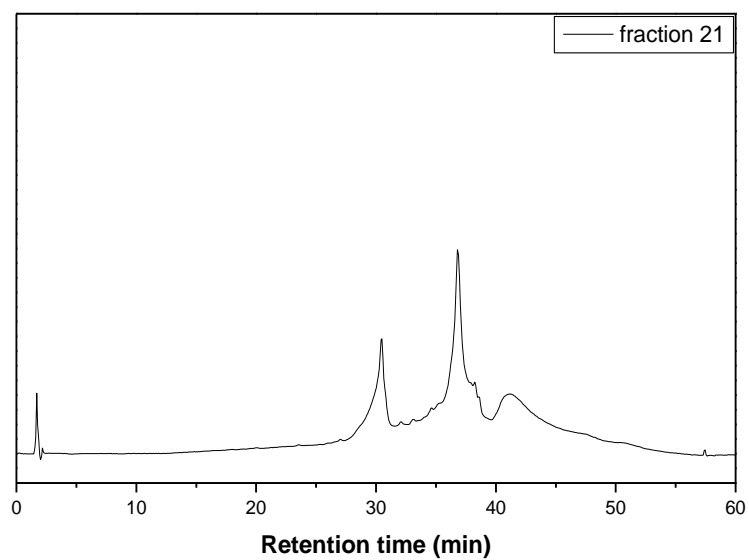
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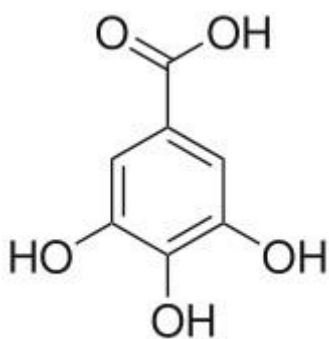
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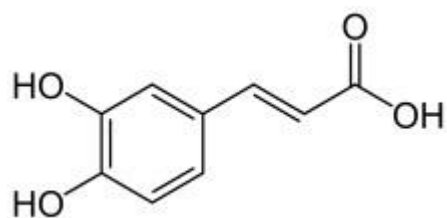
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2.4.3. LC/MS analysis and ingredient identification of separated propolis

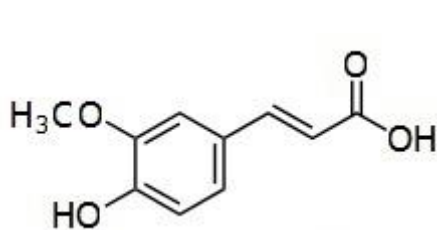
Fractioned propolis was analyzed for the identification of flavonoids and phenols using LS/MC. The phenols such as Gallic acids appeared in fraction 4, Caffeic acid did in fraction 5~8, Cinnamic acid appeared in fraction 7, and CAPE identified in fraction 13 respectively (Figure 21~26). The flavonoids such as Chrysin was identified in fraction 4, Rutin was in fraction 19, Galangin was in fraction 16~18, and Qercetin appeared in fraction 18~21 respectively (Figure 27~31).



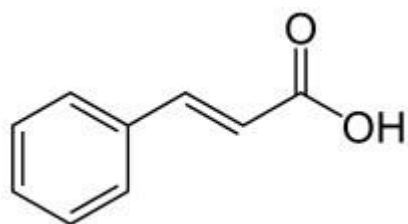
Gallic acid (mw=170.1)



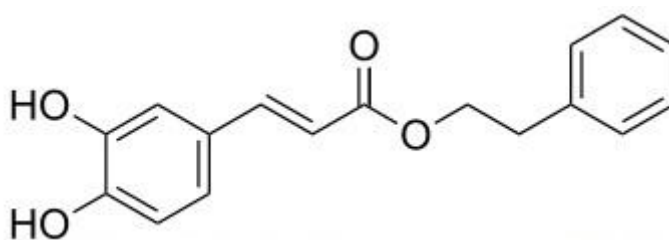
Caffeic acid (mw=180.2)



Ferulic acid (mw=194.2)

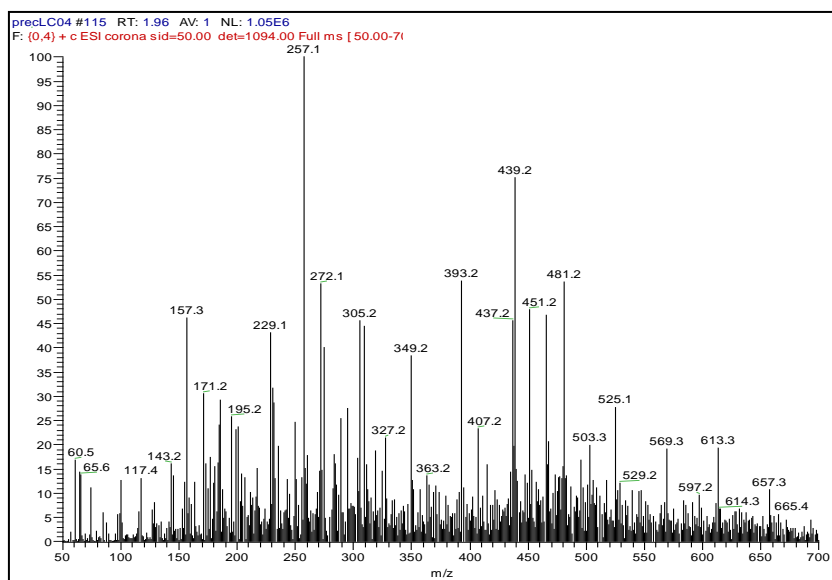


Cinnamic acid (mw=148.2)



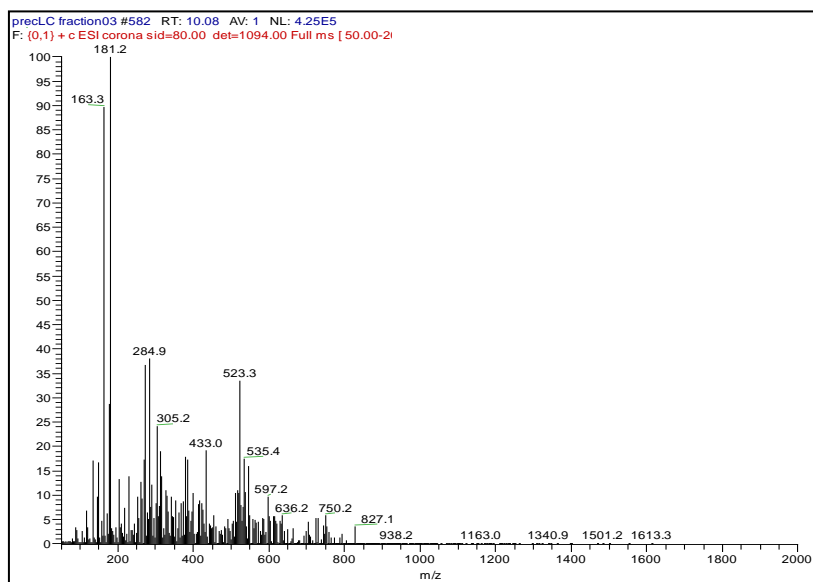
Caffeic acid phenethyl ester (CAPE, mw=284.3)

Figure 21. Structures of phenolics

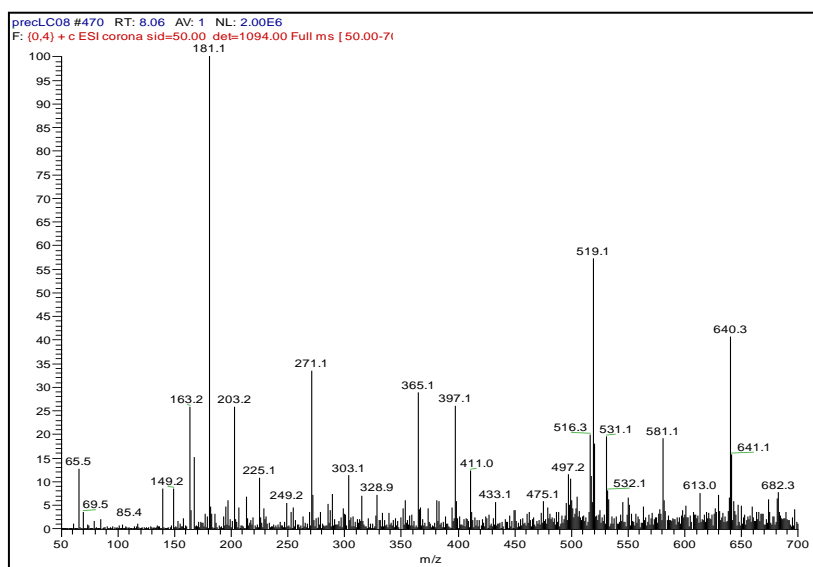


Gallic acid (171.2, fraction 4)

Figure 22. LC/MS chromatogram of gallic acid in fraction 4 of propolis
separated with prepHPLC

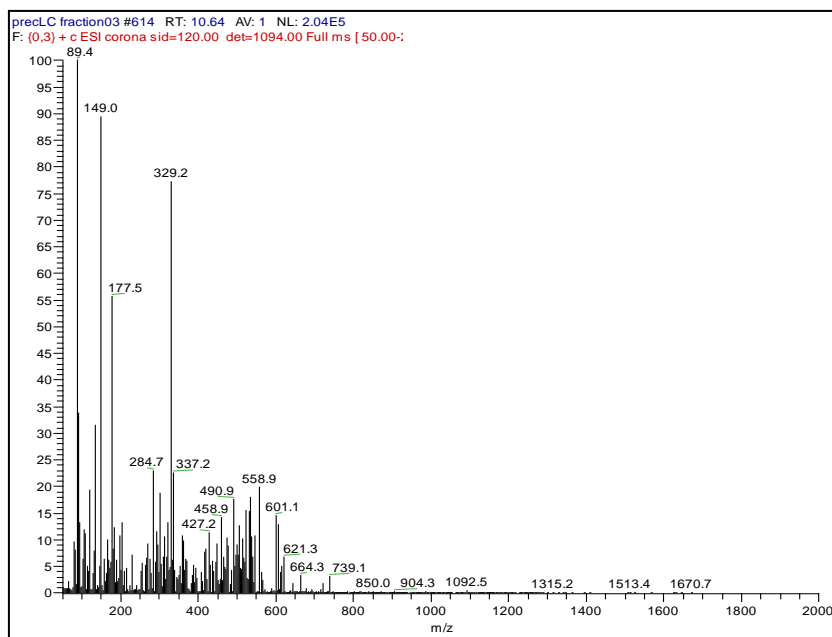


Caffeic acid (181.2, fraction 5)



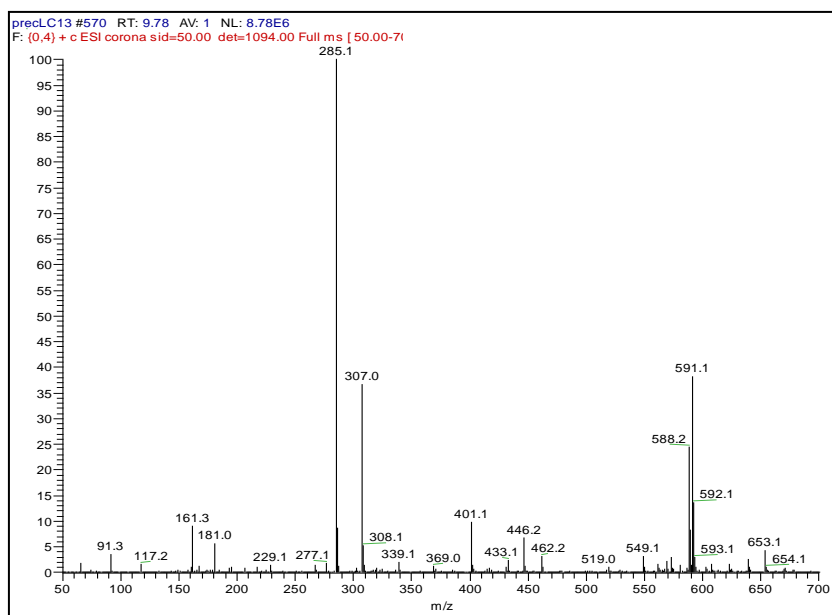
Caffeic acid (181.2, fraction 8)

Figure 23. LC/MS chromatogram of Caffeic acid (181.2) in fraction 5, 8 of propolis separated with prepHPLC



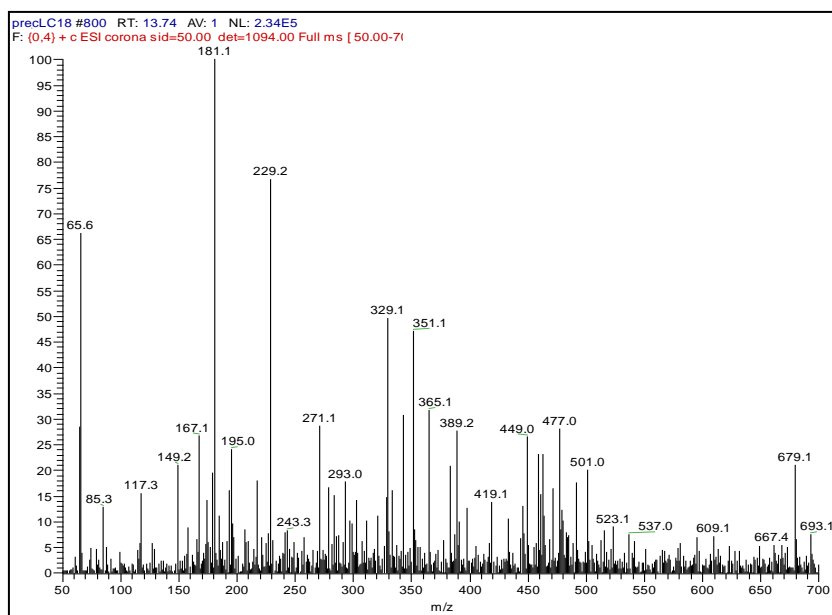
Cinnamic acid (149.0, fraction 7)

Figure 24. LC/MS chromatogram of Cinnamic acid in fraction 7 of propolis
 separated with prepHPLC



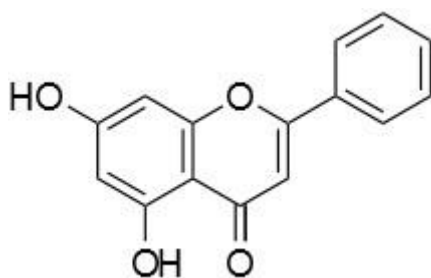
CAPE (285.1, fraction 13)

Figure 25. LC/MS chromatogram of Caffeic acid phenethyl ester (CAPE) in fraction 13 of propolis separated with prepHPLC

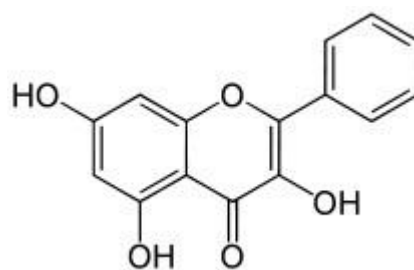


Ferulic acid (195.2, fraction 18)

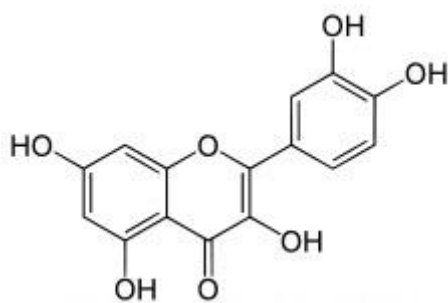
Figure 26. LC/MS chromatogram of Ferulic acid in fraction 18 of propolis
separated with preHPLC



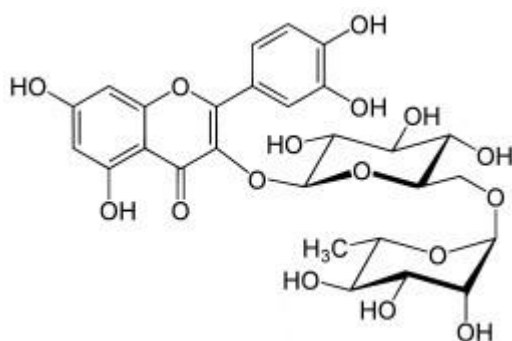
Chrysin (mw=254.2)



Galangin (mw=270.2)

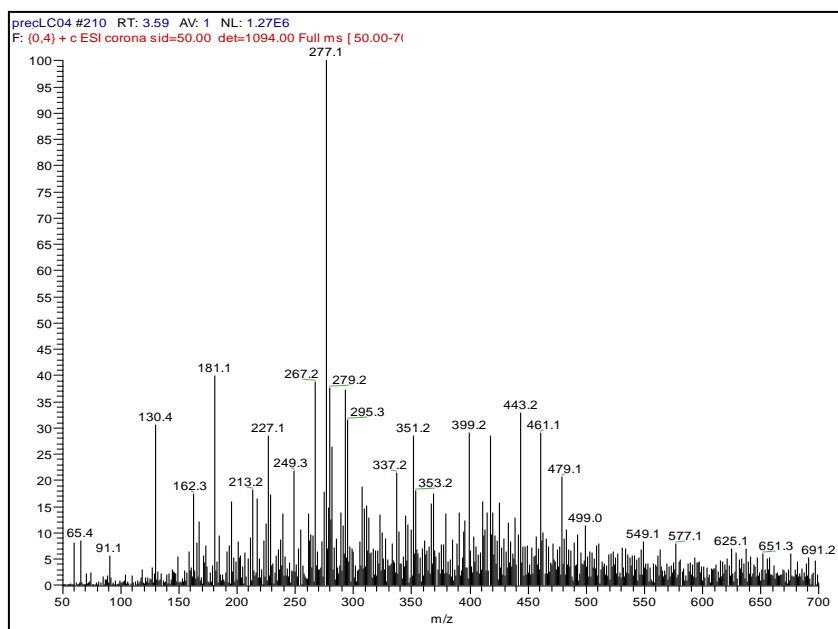


Quercetin (mw=302.2)



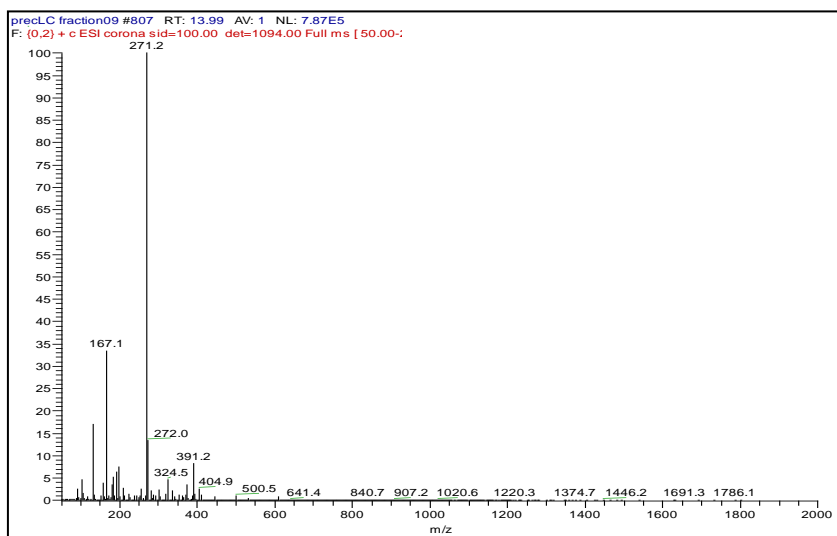
Rutin (mw=610.5)

Figure 27. Structures of flavonoids

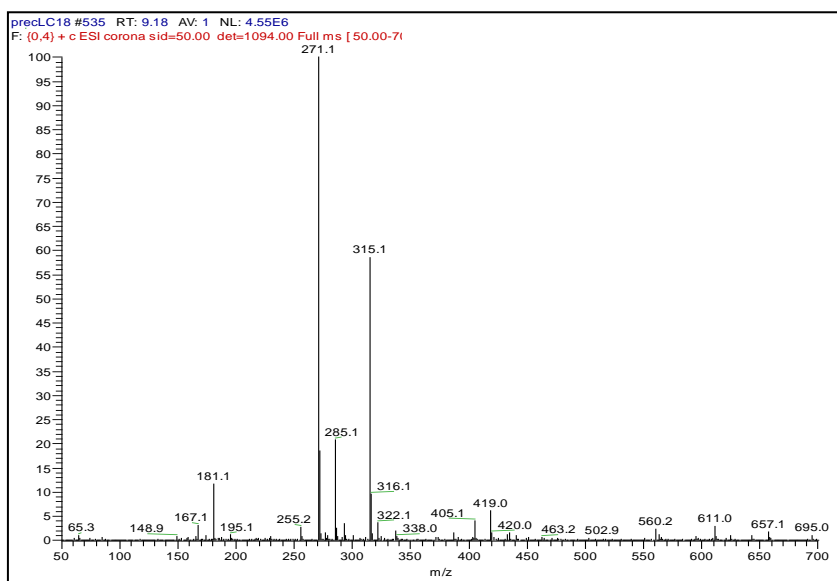


Chrysin (254.2, fraction 4)

Figure 28. LC/MS chromatogram of Chrysin in fraction 4 of propolis
separated with prepHPLC

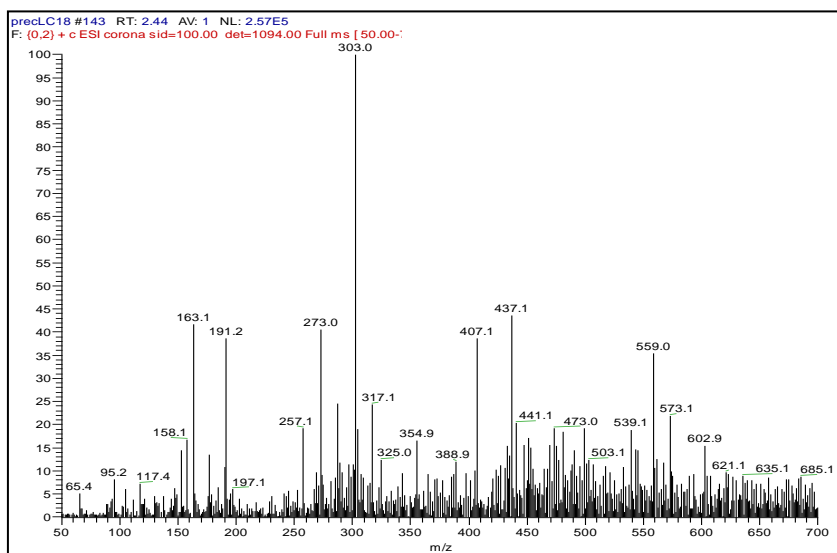


Galangin (271.2, fraction 16)

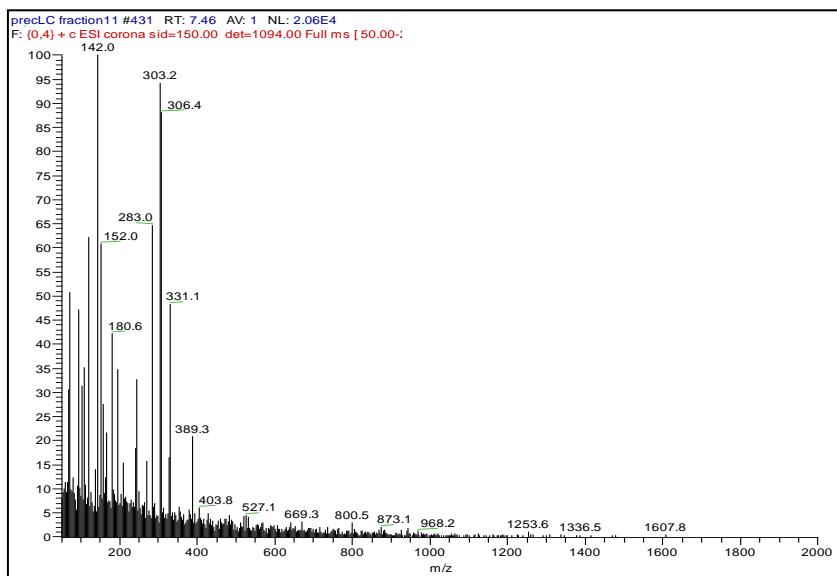


Galangin (271.2, fraction 18)

Figure 29. LC/MS chromatogram of Galangin in fraction 16, 18 of propolis separated with preHPLC

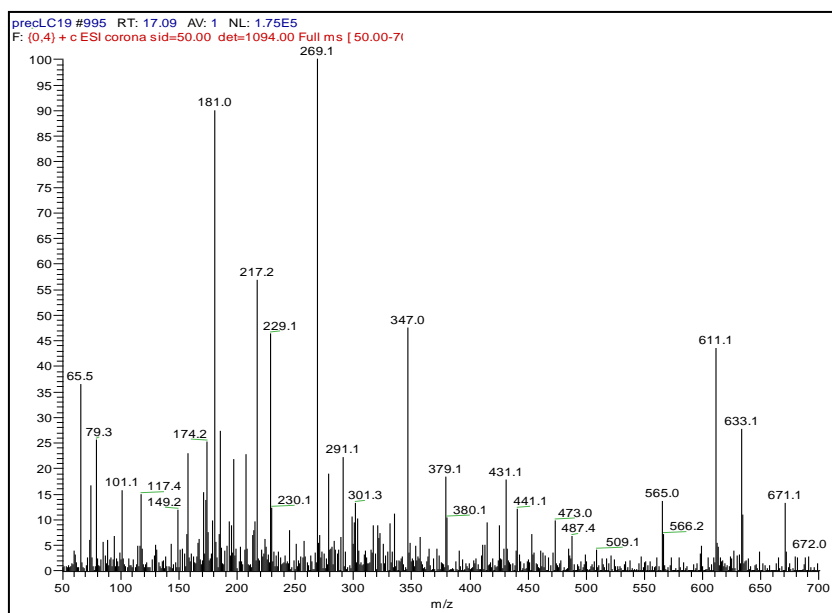


Quercetin (303.2, fraction 18)



Quercetin (303.2, fraction 21)

Figure 30. LC/MS chromatogram of Quercetin in fraction 18, 21 of propolis separated with preHPLC



Rutin(611.1, fraction 19)

Figure 31. LC/MS chromatogram of Rutin in fraction 19 of propolis separated with prepHPLC

3. Functional property of Korean propolis

3.1. Functional property range using spectrophotometer

Propolis samples from central region (Suwon, Yeongwol) showed highest absorbance value at 290nm wave length, and samples from southern region(Daegu, Hwasun) also showed highest absorbance at 290nm wave length(Figure 32). The absorbance of UV around 290nm wave length means UVB absorbing, so propolis has capability of UVA absorbance (Gregoris and Stevanato, 2010). The absorbance graph showed shoulder form around 320nm, this absorbance range is near UVA range of 320~340, so propolis can also absorb UVA (Gregoris *et al.*, 2011).

Samples from Jeju showed remarkably low absorbance value around 290nm, their absorbance value at 340~350nm range (Figure 32) was high, this means that propolis samples from Jeju have high absorbing capability of UVA.

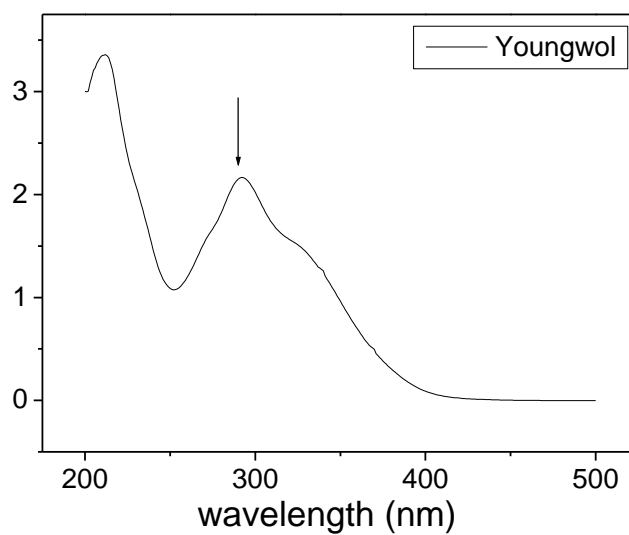
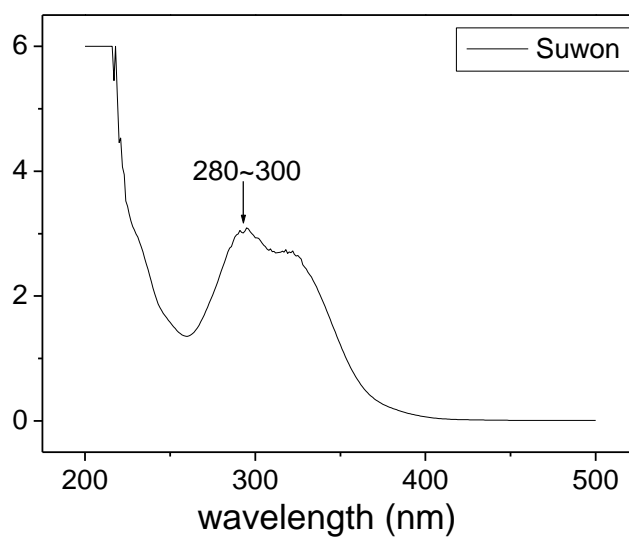


Figure 32. UV spectra of propolis extracts on Central region

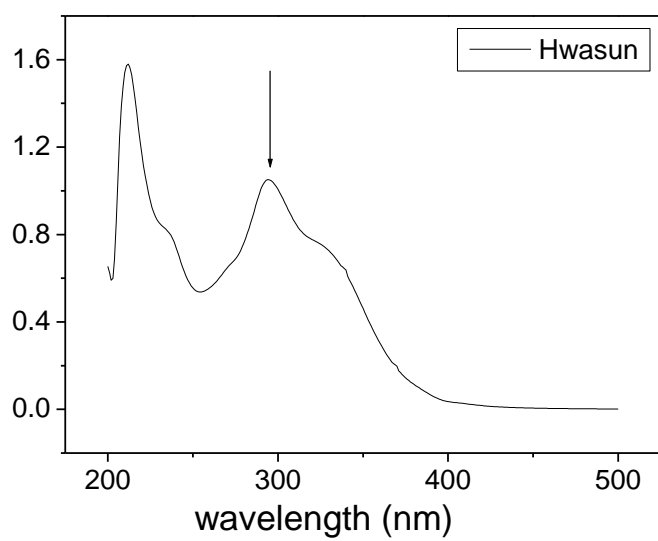
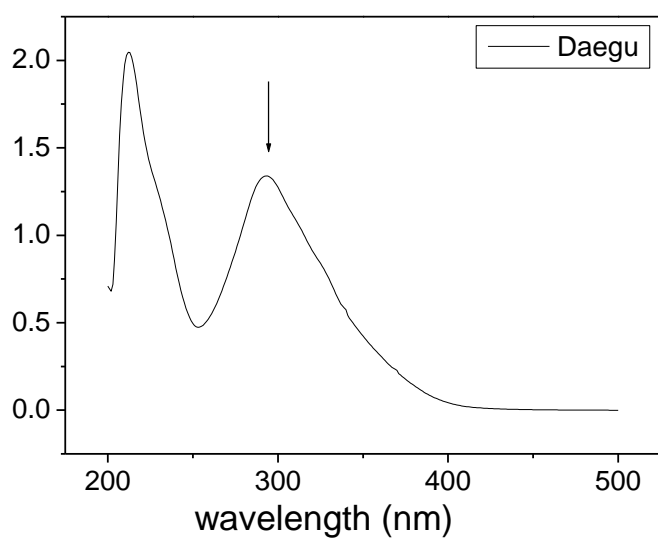


Figure 33. UV spectra of propolis extracts on Southern region

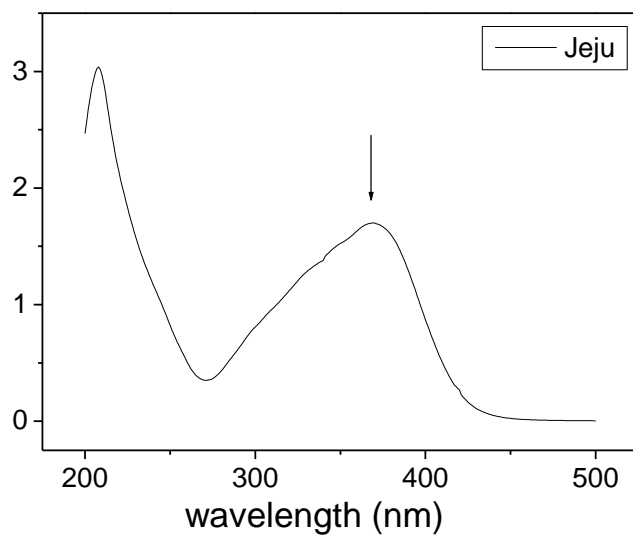
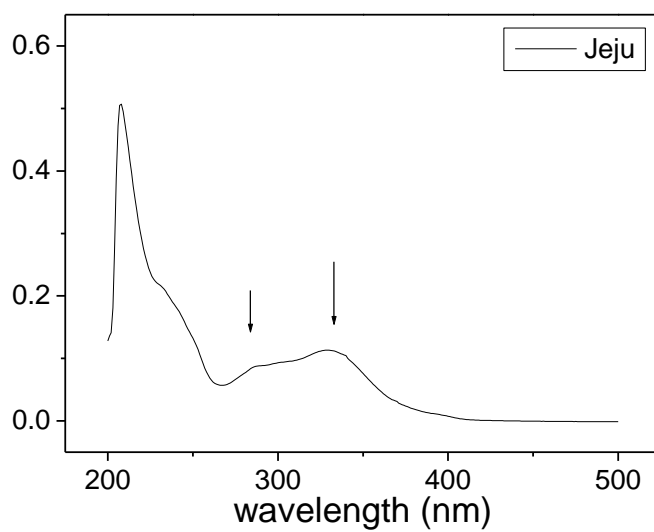


Figure 34. UV spectra of propolis extracts on Jeju island

3.2. Antimicrobial effects of propolis

3.2.1. Atimicrobial effects of intestinal microflora

All of Korean propolis samples showed good antimicrobial effect (Figure 35) to gastric ulcer inducing bacterium (*Helicobacter pylori*), and this is similar with the good antimicrobial effect of propolis from each country (Banskota *et al.*, 2001; Boyanova *et al.*, 2003). Propolis sample from Jeju showed slight antibiosis on collon bacillus (*E. coli*) but samples from Suwon and Daegu did not show antibmicrobial effects (Table 6).

3.2.2. Antimicrobial effect of various harmful microflora

3.2.2.1. Oral bacteria

Antibiosis of propolis to harmful bacteria was examined (Table 7), most propolis samples including Korean and foreign collected showed antibiosis to cavity inducing bacteria (*Streptococcus mutans*). Numbers of studies are undergoing globally on oral bacteria (Koo *et al.*, 2000; Santos *et al.*, 2002), the standard of Health Functional Food Act specified antimicrobial effects of propolis to oral bacteria by this reason.

3.2.2.2. Athelete foot fungi (yeast, fungi)

Athelete's foot fungus was treated with propolis for examining its

antifungus effects, all the samples from each region showed insignificant effect. The anti fungus effect of propolis on *Candida* fungus showed significantly different effect by region (Table 8).

3.2.2.3. Other microbes

Propolis treatment to MRSA (methicillin resistant *Staphylococcus aureus*) showed excellent antimicrobial effect (Table 9), and some samples had slight antibiosis to *Bacillus subtilis*. .

Table 6. Antibacterial effect of intestinal bacteria on propolis.

Bacteria	Sample	Suwon	Daegu	Jeju
<i>Escherichia coli</i>		-	-	+
<i>Bifidobacterium longum</i>		-	-	-
<i>Bifidobacterium adolescentis</i>		-	-	-
<i>Helicobacter pylori</i>		+++	++++	++

※ Inhibition zone diameter

+: 10~16mm

++: 16~24mm

+++: 24~32mm

++++: 32~40mm



Figure 35. Inhibition zone for propolis on *Helicobacter pylori*

Table 7. Antibacterial effects of propolis on *Streptococcus mutans*

Region	3065	3289
Gyeonggi	- ~ ++	+ ~ ++
Gangwon	+ ~ ++	+ ~ ++
Chungbuk	+ ~ ++	+ ~ ++
Chungnam	- ~ +	+
Gyeongbuk	+ ~ ++	+ ~ ++
Gyeongnam	+ ~ ++	+ ~ ++
Jeonbuk	+	+ ~ ++
Jeonnam	+ ~ ++	+ ~ ++
Jeju	- ~ +	- ~ +
China	+	+
Aldrich	+	+
Brazil	+	+

※ -: No effect, +: 0~10mm, ++: 10~14mm

KCTC 3065: *Streptococcus mutans*

KCTC 3289: *Streptococcus mutans*

Table 8. Antifungal effects of propolis on yeast and fungi

Region	7121	7728	6077	6345	6351	6586
Gyeonggi	- ~ ++	- ~ +	-	-	-	-
Gangwon	- ~ ++	-	- ~ +	-	-	- ~ +
Chungbuk	- ~ +	-	-	-	-	- ~ +
Chungnam	-	+	-	-	- ~ +	-
Gyeongbuk	- ~ ++	- ~ +	-	- ~ +	- ~ +	- ~ +
Gyeongnam	- ~ ++	- ~ +	-	- ~ +	- ~ +	-
Jeonbuk	-	-	-	-	-	-
Jeonnam	- ~ +	-	-	-	-	-
Jeju	-	-	-	-	-	-
China	-	-	-	-	-	-
Aldrich	-	-	-	-	-	-
Brazil	-	-	-	-	-	-

※ -: No effect, +: 0~10mm, ++: 10~14mm

KCTC7121: *Candida albicans*(Yeast)

KCTC7728: *Candida albicans*(Yeast)

KCTC6077: *Trichophyton mentagrophytes*

KCTC6345: *Trichophyton rubrum*

KCTC6351: *Trichophyton ferrugineum*

KCTC6586 : *Epidermophyton floccosum*

Table 9. Antibacterial effects of propolis on MRSA and *Bacillus subtilis*

Region	MRSA	<i>Bacillus subtilis</i>
Gyeonggi	+ ~ ++	+
Gangwon	- ~ ++	- ~ +
Chungbuk	+ ~ ++	+ ~ ++
Chungnam	+ ~ ++	- ~ +
Gyeongbuk	+ ~ ++	+
Gyeongnam	+ ~ ++	- ~ +
Jeonbuk	+ ~ ++	+
Jeonnam	+	+
Jeju	- ~ +	- ~ +
China	+	-
Aldrich	++	+
Brazil	++	+

※ -: No effect, +: 0~10mm, ++: 10~14mm

※ MRSA : methicillin resistant *Staphylococcus aureus*

3.2.3. Propagation effect on beneficial intestinal microflora

Propagation effect of propolis to intestinal microbes were examined (Figure 36), treatment of Suwon sample increased propagation with 5mg/6ml treatment, but 25mg/6ml treatment caused 20% decrease. Also the propagation increased until 5mg/6ml treatment of Hwasun sample, and decreased to less than 1/3 with 25mg/6ml treatment. Jeju sample showed increased propagation with 5mg/6ml, and showed 30% decrease with 25mg/6ml treatment. Each propolis sample showed increase of propagation with lower concentration treatment, on the contrary they induced propagation decrease with higher concentration.

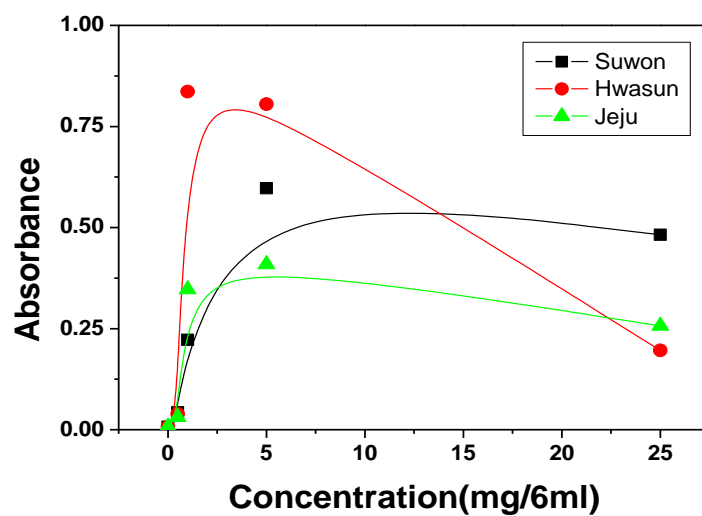


Figure 36. Propagation effects of propolis on *Bifidobacterium longum*

3.3. Anti-oxidant effect of propolis

3.3.1. Anti-oxidant effect of propolis in vitro

3.3.1.1. Free radical scavenging effect by DPPH method

To examine anti-oxidation effect of propolis, free radical scavenge effect of DPPH was investigated (Figure 37), samples from Central and Southern region showed increase of anti-oxidation concentration rise to 100ug, and the effect was maintained with 500 and 1,000ug concentration. Samples from Jeju showed continuous anti-oxidation increase, and its effect was lower than samples from Central and Southern region. And Jeju sample showed similar tendency of anti-oxidation effect increase according concentration rise (Choi *et al.*, 2006).

3.3.1.2. OH radical scavenging activity of 2-Deoxy-D-ribose

The OH radical scavenging activity of propolis was measured (Figure 38), propolis samples from Central region showed tendency of activity increase by their concentration rise, the samples from Southern regions showed 37~50% of activity and concentration rise did not increase anti-oxidation effect significantly. Jeju sample's activity was 35% with 100ug concentration, and was over 85% with 1000ug concentration. All samples showed tendency of OH radical scavenging effect increase by concentration rise.

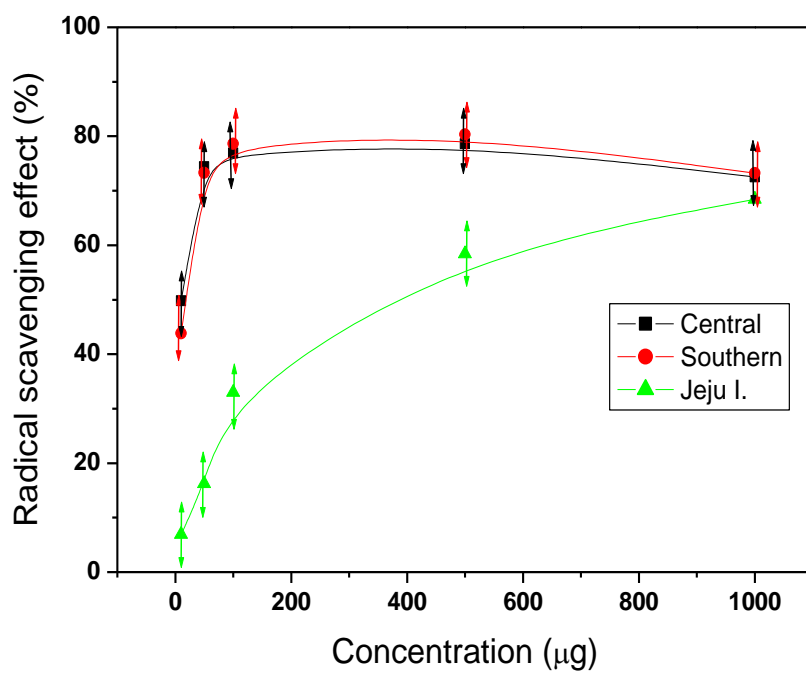


Figure 37. Free radical scavenging effects of DPPH with propolis collected in Korea

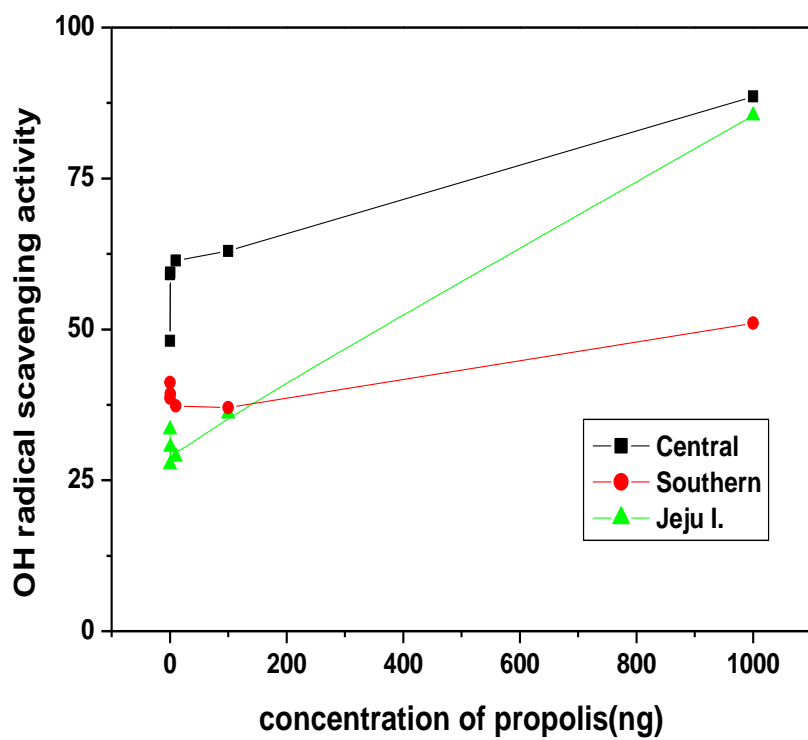


Figure 38. OH radical scavenging activity of 2-Deoxy-D-Ribose on propolis collected in Korea

3.3.1.3. Radical scavenging effect Ferric thiocyanate method

The radical scavenging effect of propolis was examined using ferric thiocyanate method (Figure 39), samples from Central region showed over 80% anti-oxidation effect regardless of sample concentration, and samples from Southern region showed increase of effect as the concentration rises. Jeju samples showed radical scavenging effect at low concentration but its scavenging effect decreased to zero by concentration rises.

3.3.1.4. Free radical scavenging effect by soybean lipoxygenase method

The effect of propolis free radical scavenging was determined using soybean lipoxygenase method (Figure 40), propolis from Central region has similar tendency with vit. C, samples from Southern region showed highest effect at 500ug/ml concentration, Jeju samples showed highest value at 250ug/ml, and their effect decreased by concentration rise.

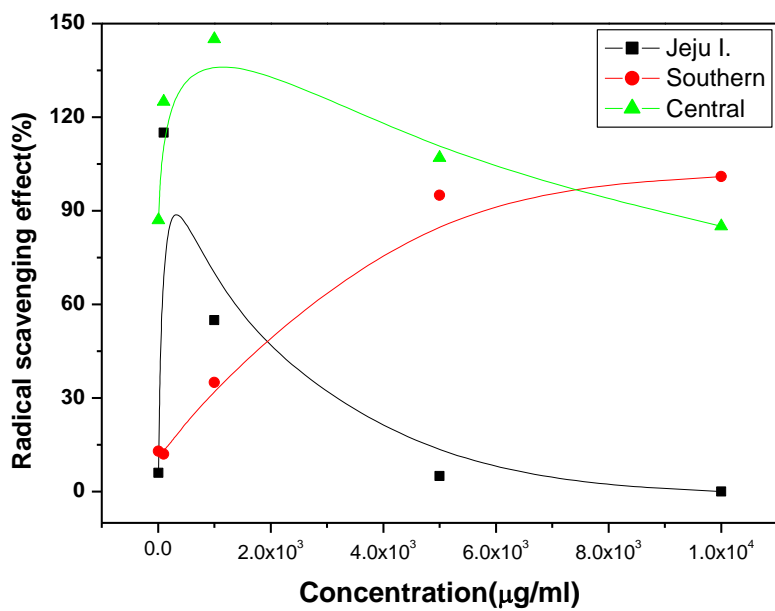


Figure 39. Free radical scavenging effects by ferric thiocyanate method on propolis collected in Korea

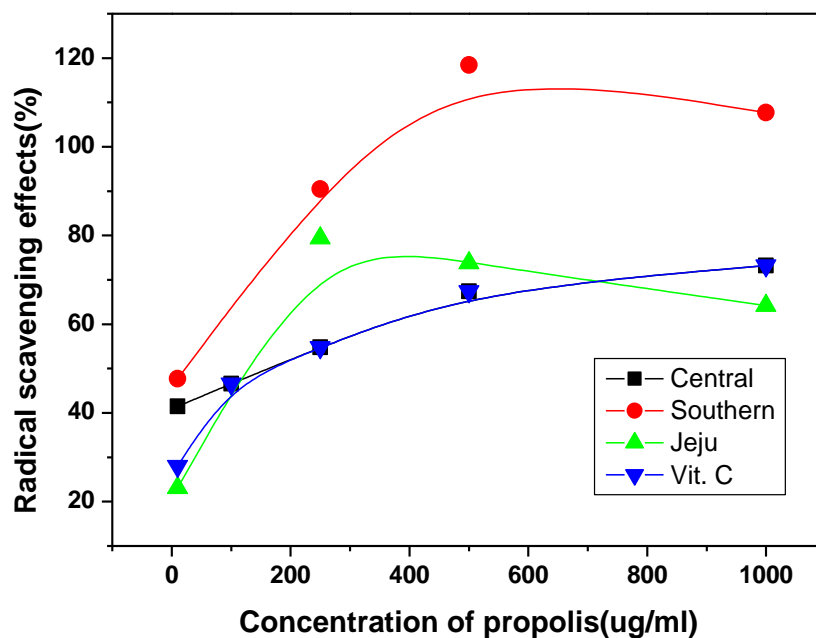


Figure 40. Free radical scavenging effects by soybean lipoxygenase method on propolis collected in Korea

3.3.2. Anti-oxidant effects of propolis in vivo

3.3.2.1. Total glutathione content

Total glutathione content in liver tissue was determined (Figure 41), propolis treated sample (120~150%) showed 20~50% increase of total glutathione content than no-treated (90~105%).

3.3.2.2. Lipoperoxide content of liver tissue

The determination of lipoperoxide content in liver tissue showed 15~20% decrease of propolis treated sample compared to control (Figure 42), anti-oxidation of propolis was excellent considering total glutathione and lipoperoxide content.

3.3.2.3. Glutathione peroxidase activity (GSH-Px)

Activity of peroxidase to gultathione was measured (Figure 43), the activity had tendency of gradual increase by propolis concentration rise. Propolis from Central and Southern region showed more than 10 times of activity than sample from Jeju. This suggests that propolis significantly contribute to increase of peroxidase.

3.3.2.4. Glutathione S-transferase activity(GST)

The result of Glutathione S-transferase activity (unit) measurement (Figure 44) showed approximately 2 times more activity of Central region samples than other region originated samples. The activity decreased by propolis concentration rise.

With these results of in vivo and in vitro experiments, excellent anti-oxidation effect of propolis was confirmed, and showed consistency with the studies identifying various anti-oxidation effects of propolis (Arjun *et al.*, 2000; Fang *et al.*, 2000; Isla *et al.* 2001; Russo *et al.*, 2002; Kolankaya *et al.*, 2002; Hamasaka *et al.*, 2004; Kumazawa *et al.*, 2004; Ozen *et al.*, 2004; Shimizu *et al.*, 2004).

All the results from above experiments revealed excellent anti-oxidation effect of propolis, and this means propolis has high potential for using an effective health functional food resource.

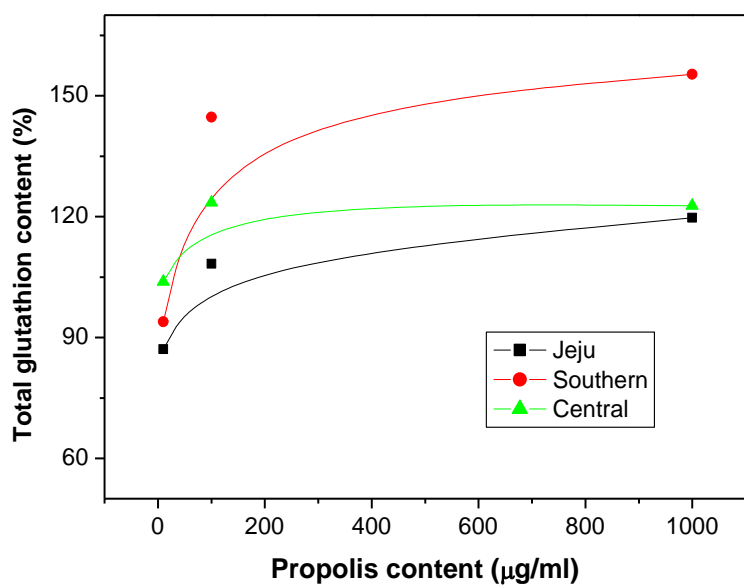


Figure 41. Total glutathione contents (%) in liver according to propolis concentrations.

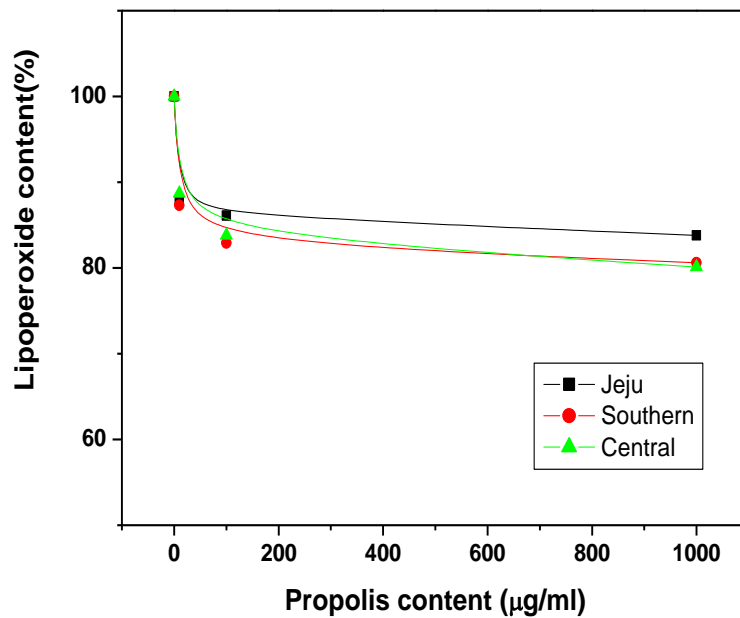


Figure 42. Lipoperoxidase content in liver treated with propolis concentrations (ug/ml)

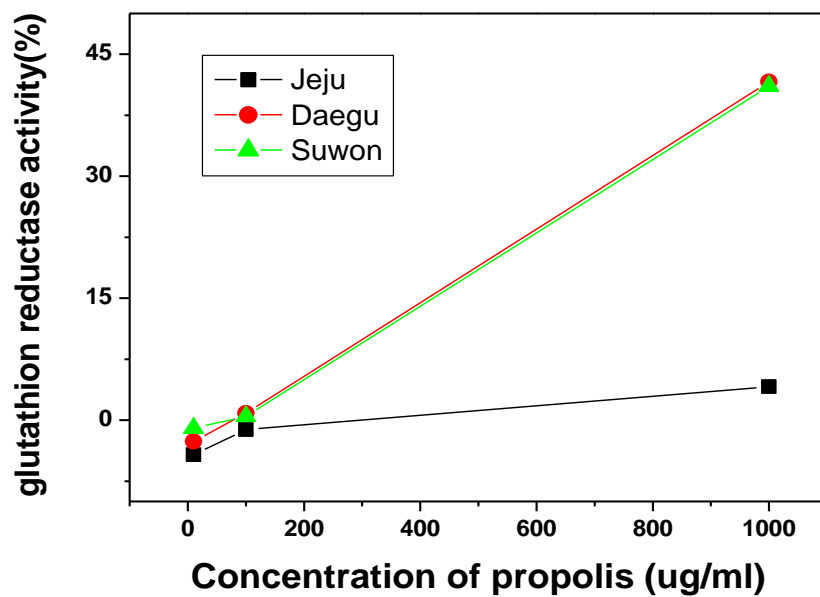


Figure 43. Glutathione peroxidase activity in liver according to propolis concentration (ug/ml)

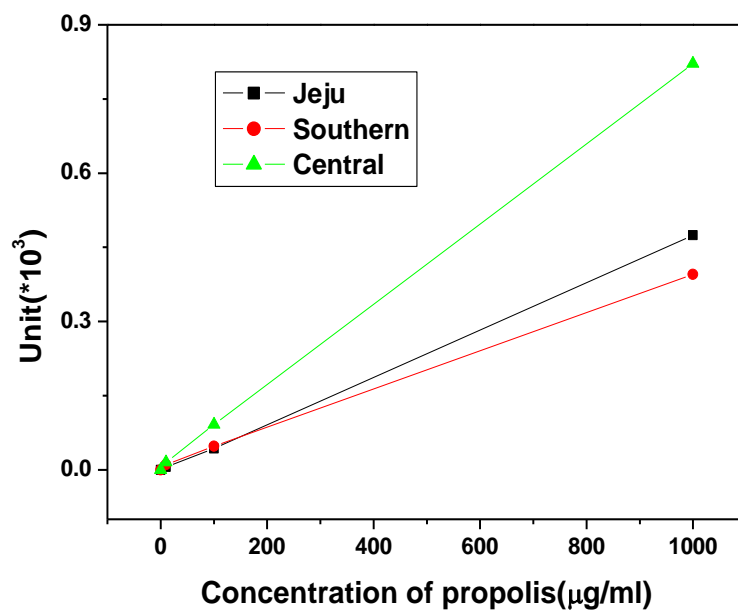


Figure 44. Glutathione S-transferase activity(unit) in liver according to propolis concentration (ug/ml)

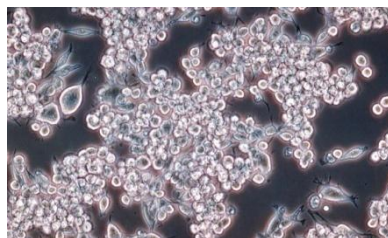
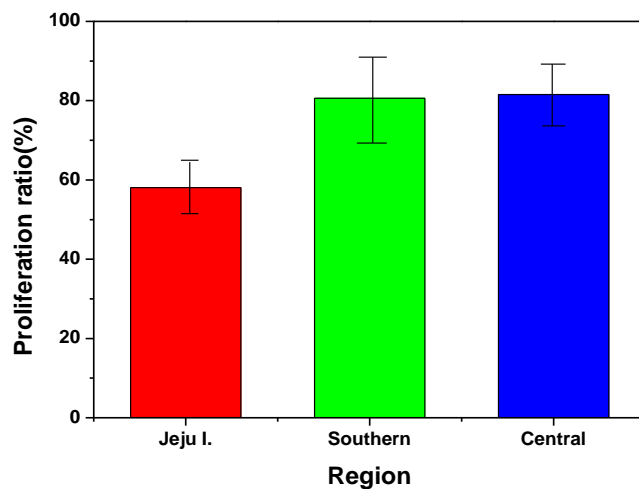
3.4. Stomach cancer inhibition effect of propolis

3.4.1. Cell propagation ratio (MTT assay)

The protection effect of propolis on stomach was investigated (Figure 45, 46), propolis samples from Jeju showed lower cell survival ratio to RAW264.7 and SNU484 cell lines than those from other regions. So Jeju sample is effective to inhibit the propagation of inflammation cell and cancer cell.

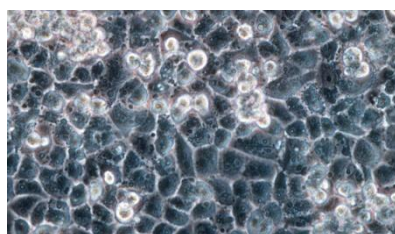
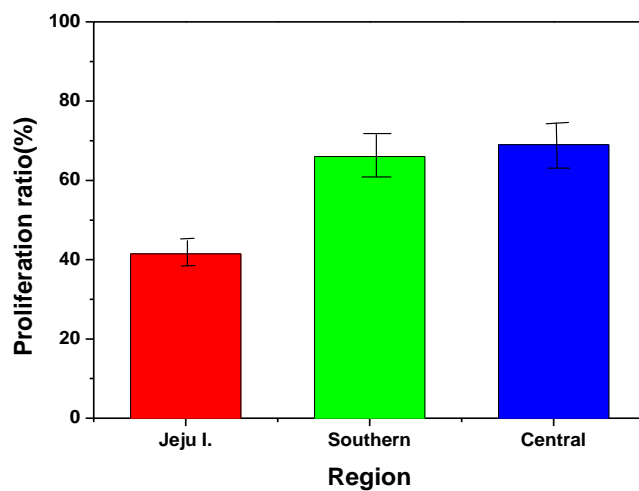
3.4.2. Protein expression phase

The expression of E-cadherin protein and β -catenin protein which express with stomach cancer occurrence was measured using SNU484 cell line originated from stomach cancer cell (Figure 47, 48), the protein expression decrease was identified after propolis treatment.



RAW 264.7 cell line

Figure 45. Proliferation ratio with MTT assay (sample : 100ug) for RAW 264.7 cell line.



SNU 484 cell line

Figure 46. Proliferation ratio with MTT assay (sample : 100ug) for SNU484 cell line.

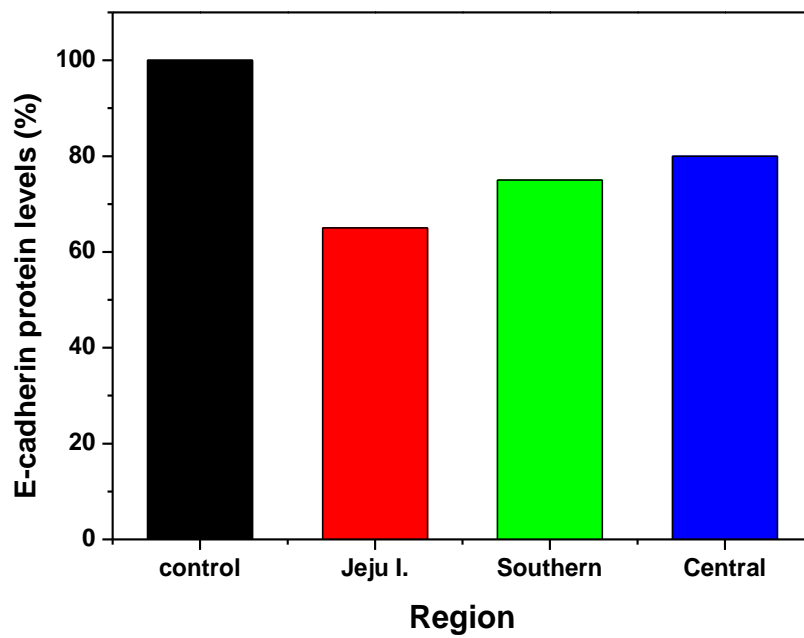


Figure 47. E-cadherin protein expression levels with SNU484 cell line.

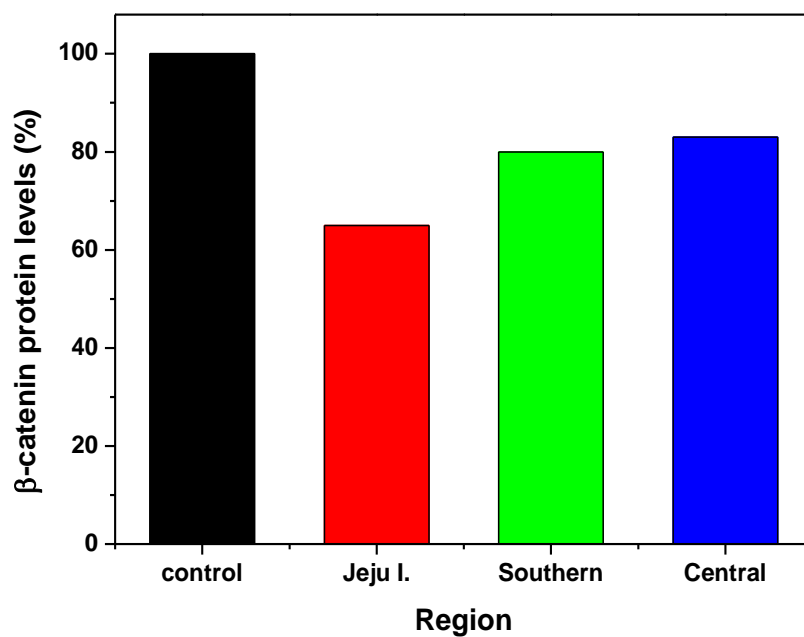


Figure 48. B-catenin protein expression levels with SNU484 cell lines.

3.4.3. Stomach cancer inhibition effect of propolis (in vivo)

Stomach related protection effect of propolis was investigated using nude mouse by observing body weight and tumor size changes after injecting cancer cell and feeding propolis (Figure 49, 50). The normal group showed continuous body weight increase until the end of experiment, both control and treatment group showed body weight loss after cancer cell injection and there were no significant difference between control and treatment group (Figure 51).

There was no significant tumor size difference between control and treatment group until 14 days after injection, and treatment group's tumor size showed significant decrease 21 days after injection, the higher propolis concentration was the more decrease of tumor size was. Negative tumor size was observed with lower concentration propolis feeding (Figure 52). This suggest that the amount of propolis affects tumor size decrease.

The expression of COX-2 in propolis fed and control group was compare at mRNA level, COX-2 expression was low at low concentration, and there was no significant difference between medium, high concentration and control group. The decrease COX-2 was identified from propolis fed group (Figure 53).

These results confirm that propolis ingestion affects stomach cancer cell growth.



Figure 49. Nude mouse and MKN45 cell line for this work.



Figure 50. Body feature changes after injection on MKN45 cell lines

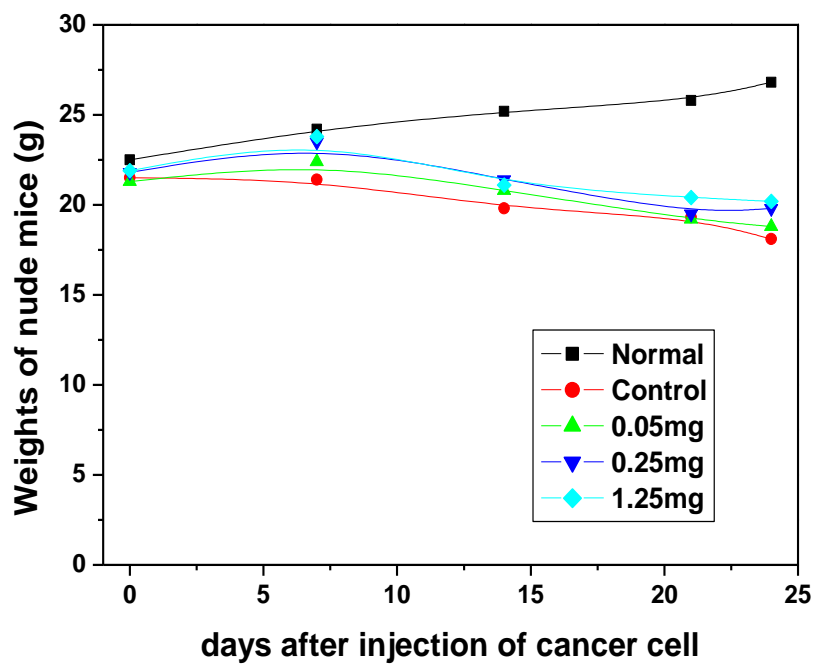


Figure 51. Body weight changes of nude mice after injection of cancer cell lines (t test, $p < 0.05$)

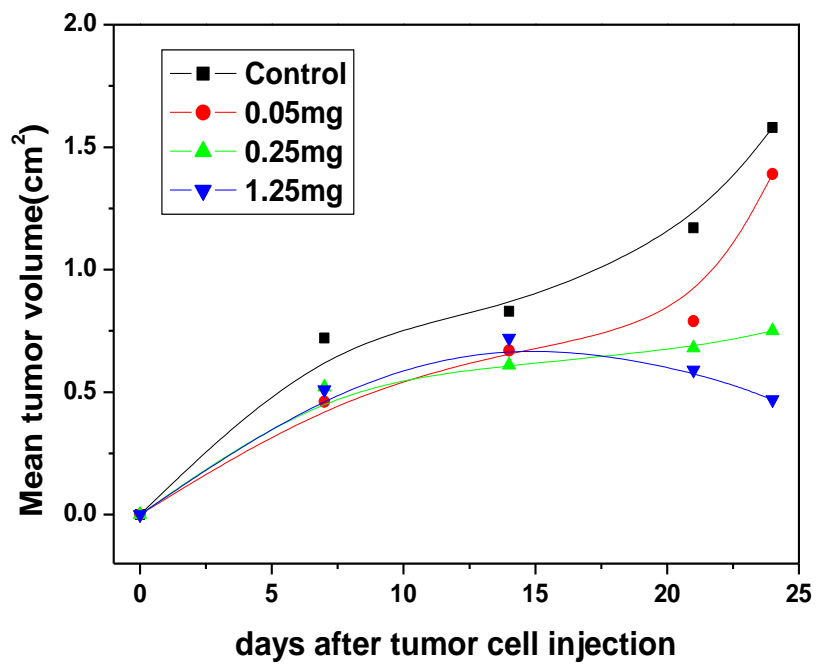


Figure 52. Mean tumor volume changes after tumor cell injection.

(t test, $p < 0.05$)

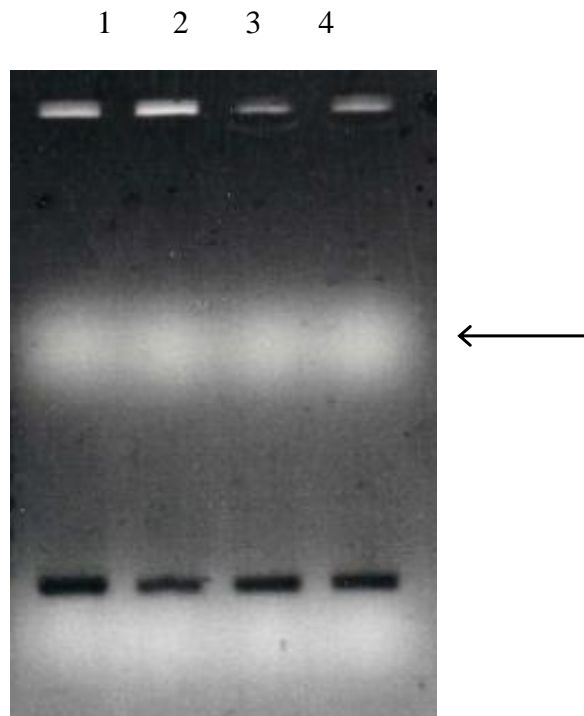


Figure 53. RT-PCR for COX-2 expression.

1. Control 2. 0.05mg 3. 0.25mg 4. 1.25mg

IV. Discussion

Propolis is a sticky material made from bee collected plant growth point protection secretion or resin mixed with bee saliva enzyme, used for keep bee colony safety by applying inside of bee hive, and it is consisted of about 50% of resin and aromatic, 25% of bee wax, 10% of essential oil, pollen and mineral.

1. Characteristics of propolis extraction with ethanol

The extraction yield and total flavonoid content were excellent at 70% ethanol (available for spirit with edible use). And this is consistent with the result (Kujumgiev *et al.*, 1999; Santos *et al.*, 2002; Lu *et al.*, 2005; Mani *et al.*, 2006) which used 70~100% for propolis extraction. Total phenolic content was highest extracted at 50~60% EtOH, and polyphenol extraction yield was highest about 50% EtOH. This suggests that propolis extraction is more efficient than just using alcohol spirit when proper amount of water is mixed with ethanol. Twelve hour was enough for full extraction of propolis, and extraction temperature was no matter except freezing condition.

2. General characteristics propolis collected by time and origin (Korean & foreign)

Propolis sample were collected with every 2 weeks, collection amount and ingredient change were monitored analyzed. Average propolis collection amount during summer (beginning of June ~ end of August) was 87g occupying 87% of annual collection (100g). Total content of flavonoid and phenolic by collection time was measured, total flavonoid content was 7~8%, total phenolic content was 16~20%, and these contents were maintained without significant difference. When compared with foreign data, Korean propolis showed similar values (Teixeira *et al.*, 2010).

Ingredient change by collection time was identified from HPLC analysis which showed different peak height and position (Simões- Ambrosio *et al.*, 2010).

Most of Korean propolis collected from various region showed more than 5% of total flavonoid content (standard of Health Functional Food Act). All inland collected propolis fulfilled this standard, but propolis from Jeju showed pretty low content to fulfill the standard. The inland propolis sample showed more than 20% total phenolic content (Kumazawa *et al*, 2004; Bonvehí & Gutiérrez, 2012) and Jeju sample showed about 15% content.

Some amino acids in propolis were identified from amino acid content

analysis, and different amino acids were detected by collection region. Jeju propolis sample showed brighter color than other regions, color of other samples were various from yellow to brown (Burdock, 1997), and there was no indigenous color by region. Heavy metal content of propolis was examined, content of lead was less than standard (5ppm), arsenic and mercury were negative. Slight amount of minerals which are necessary for metabolism were contained. Crude lipid content of propolis from Central and Southern region were average 38%, and that of Jeju sample was 24%.

Major ingredients of propolis were identified by comparing HPLC results with standards. Most of 6 standard ingredients were identified from Korean propolis. Samples from Yeongwol and Jeju had different peaks, and samples from Brazil and China also had indigenous peaks. These different peaks suggests reflection of different woody and herbal plant species characteristics of originated region (Alencar *et al.*, 2007; Guo *et al.*, 2011; Kumazawa *et al.*, 2013. Collected propolis samples were fractioned by prepHPLC and analyzed by HPLC and LC/MS. Unique ingredients were identified form each fraction.

3. Functional property domain using spectrophotometer

The absorbance range of propolis was examined to investigate the functional domain of Korean propolis using UV/VIS spectrophotometer. Most of propolis sample showed highest absorbance about 290nm range, and

shoulder form appeared around 320nm wave length. This is overlapped with UVA range of 320~340nm, and suggests UVA absorbing capability of propolis (Gregoris *et al.*, 2010; Gregoris *et al.*, 2011).

4. Antimicrobial effect of propolis

Propagation suppression effect of propolis on intestinal microbes were examined, all Korean propolis showed excellent suppression effect to gastric ulcer inducing bacterium *Helicobacter pylori*, and this is similar with the good antibiosis of propolis from each country (Banskota *et al.*, 2001; Boyanova *et al.*, 2003).

Propolis sample from Jeju showed slight antimicrobial effect on collon bacillus (*Escherichi coli*). The investigation of antibiosis to various harmful microorganisms showed antibiotic effect of propolis to oral bacterium (*Streptococcus mutans*). Numbers of studies are undergoing globally on oral bacteria (Koo *et al.*, 2000; Santos *et al.*, 2002), the standard of Health Functional Food Act specified antibiosis of propolis to oral bacteria by this reason. Propolis also has high antibiosis to MRSA. This suggests that propolis is effective to bacteria which has antibiotic resistance as a natural substance.

5. Anti-oxidant effect of propolis

The anti-oxidation effect of propolis was investigated by various way, propolis concentration rise induced increase of anti-oxidation effect, but the effect decreased at 1000 μ g concentration on the contrary. The content of glutathione increased and lipoperoxide content decreased in vivo by the rise of propolis concentration. With these results of in vivo and in vitro experiments, excellent anti-oxidation effect of propolis was confirmed, and showed consistency with the studies identifying various anti-oxidation effects of propolis (Arjun *et al.*, 2000; Fang *et al.*, 2000; Isla *et al.* 2001; Russo *et al.*, 2002; Kolankaya *et al.*, 2002; Hamasaka *et al.*, 2004; Kumazawa *et al.*, 2004; Ozen *et al.*, 2004; Shimizu *et al.*, 2004).

6. Inhibition of stomach cancer of propolis

Based on excellent effect of propolis on gastric ulcer, stomach protection effect of propolis was identified by examining survival ratio of RAW264.7 and SNU484 cell lines after propolis treatment. Treatment of Jeju propolis sample showed least cell survival ratio. The SNU484 cell line originated from stomach cancer cell was used to test the expression of E-cadherin and B-catenin protein appeared by occurrence of stomach cancer. Propolis treatment showed decrease of these proteins expression. Body weight and tumor size of cancer cell injected and propolis fed mice were monitored, tumor size showed

decreasing tendency by propolis concentration rise. Awale *et al.* (2008) and Chen *et al.* (2013) reported CAPE suppresses growth of stomach cancer cell propagation by inhibiting metastasis of stomach cell line to epithelial mesenchymal transition.

This study identified outstanding capability of propolis to antibiosis effect on gastric ulcer inducing bacteria (*Helicobacter pylori*), cell propagation inhibition and tumor atrophy effect on cancer cell line, even its anti-oxidation effect was registered in the Health Functional Food Act for general use. It is considered that propolis can be developed to health functional food with stomach protection as well as anti-oxidation effect.

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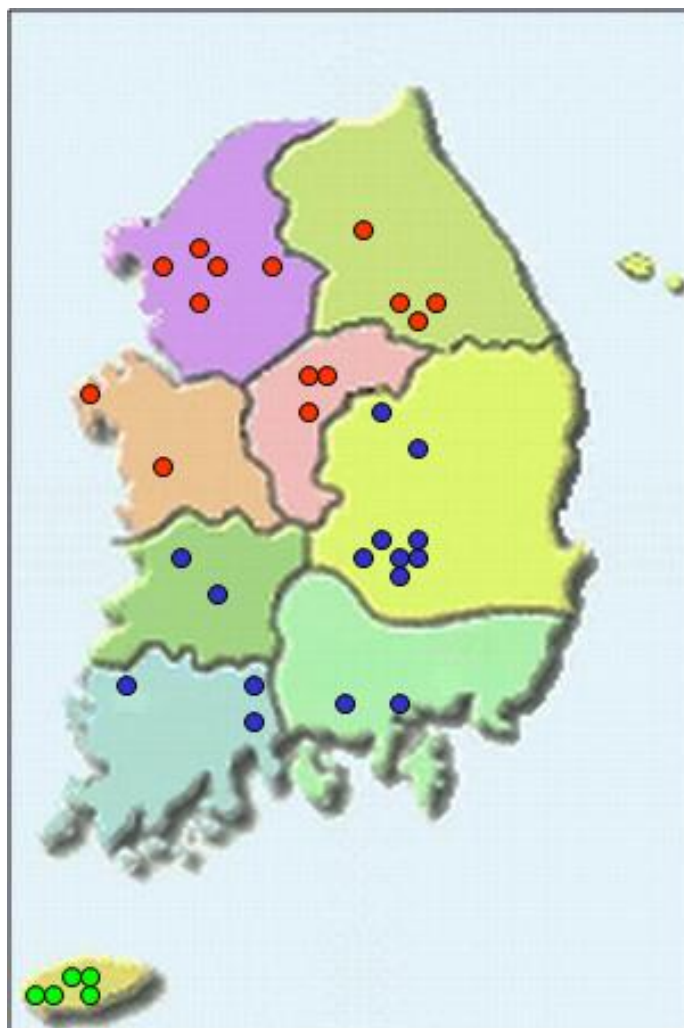
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Appendix. Area of propolis collection



- Central region: Gyeong-gi do, Gang-won do, Chung-cheong bukdo, Chung-cheong namdo
- Southern region: Gyeong-sang namdo, Gyeong-sang bukdo, Jeolla namdo, Jeolla bukdo
- Jeju island

국문 초록

한국산 프로폴리스의 항산화, 항균, 항암 효과에 관한 연구

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프로폴리스는 끈적이는 교질성 물질로서 꿀벌들이 수목류의 생장점 보호물질을 수집 타액과 혼합하여 만드는 것으로 벌통내부에 발라 벌들의 안전을 위하여 사용하는 물질이다. 프로폴리스는 50% 내외의 수지물질과 방향성 물질, 25%내외의 밀랍, 10%내외의 정유, 꽃가루, 무기물 등으로 구성되어 있다.

이 연구에서는 프로폴리스는 주로 식품으로 이용하기 때문에 에탄올 추출 특성을 확인하였는데, 에탄올의 농도는 70%이상일 때 추출 수율과 유효성분인 총플라보노이드 함량에서 우수한 결과를 얻었다. 추출시간은 하루이상이면 추출이 되었으며, 추출온도는 냉장 조건 이상에서는 추출하는데 문제가 없었다.

프로폴리스를 2주 간격으로 수집하여 시기에 따른 수집량과 성분 변화를 확인한 결과, 수집량은 여름(6월 초 ~ 8월 말)에 87g

으로 전체(100g)의 87%를 차지하였다. 시기에 따라 수집한 프로폴리스의 총플라보노이드와 총페놀함량을 확인한 결과 총플라보노이드는 7~8%, 총페놀함량은 16~20%범위에서 큰 차이 없이 유지되었다.

국내에서 다양한 지역에서 수집한 프로폴리스에 대하여 총플라보노이드함량을 확인해 본 결과 제주 지역(1%이하)을 제외하고는 대부분 5% 이상을 나타내었다. 총페놀함량은 20%이상의 값을 나타내었으며, 제주산도 15%정도의 값을 나타내었다. 프로폴리스에서 아미노산의 함량을 확인해본 결과 다양한 아미노산이 관찰되었다. 프로폴리스의 색상은 대부분 황색에서 갈색으로 나타났으며, 제주 지역은 다른 지역에 비하여 열게 나타났다. 프로폴리스의 중금속 함량은 낮은 기준치(5ppm)이하로 나타났으며, 비소, 수은은 전혀 검출되지 않았고, 신진대사에 필요로 하는 미네랄(Zn, Cu, Ni)은 미량이 함유되어 있었다. 프로폴리스에 함유되어 있는 조지방은 중부, 남부 지역은 평균 38% 정도의 값을 나타낸 반면, 제주지역은 24%를 나타내었다. 수집한 프로폴리스를 HPLC로 분석하여 표준품과 비교한 결과 프로폴리스가 갖고 있는 주요물질(Gallic acid, naringenin, quercetin, apigenin, chrysin, galangin)을 확인하였다.

위궤양유발균(*Helicobacter pylori*)에 대하여 항균효과를 확인한 결과, 모든 국내산 프로폴리스에서 우수한 항균효과를 나타내었으

며, 구강균(*Streptococcus mutans*)에 대하여도 우수한 항균효과를 나타내었다. 장내균(유산균)에 대하여 증식효과를 확인해 본 결과는 농도가 낮을 때 높은 효과를 나타내었다.

프로폴리스의 항산화 효과를 여러 가지 방법으로 탐색한 결과 프로폴리스의 농도가 높아지면 항산화 효과도 높아지는 것으로 나타났다으나 1000 μ g에서는 오히려 떨어지는 것으로 나타났다. in vivo에서는 glutathione 함량은 증가하고 과산화지질은 감소하였으며, 프로폴리스의 농도가 증가할수록 더 높은 효과를 나타내었다.

누드마우스를 이용하여 종양을 유발시키고 프로폴리스를 섭취하게 해서 몸무게 변화와 종양의 크기 변화를 확인한 결과, 종양의 크기는 프로폴리스의 농도가 증가할수록 작아지는 경향을 나타내었다.

프로폴리스의 기능성으로 항산화 효과는 일반적이지만 프로폴리스가 위궤양유발균(*Helicobacter pylori*)에 대하여 우수한 항균효과를 가지고 있고, 위암세포주에 대하여도 세포증식율을 저하시키고 종양의 크기도 줄일 수 있다는 것을 보여주는 결과로 프로폴리스를 건강기능식품으로서 항산화효과 뿐만 아니라 위관련 보호기능이 있는 건강기능식품으로 발전할 수 있을 것으로 사료된다.

주요어 : 프로폴리스, 항산화효과, 항균효과, 항위암효과
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